(12) UK Patent Application (19) GB (11) 2 316 082 (13) A

(43) Date of A Publication 18.02.1998

(21) Application No 9616957.8

(22) Date of Filing 13.08.1996

(71) Applicant(s)
Finnfeeds International Limited

(Incorporated in the United Kingdom)

PO Box 777, MARLBOROUGH, Wilts, SN8 1XN, United Kingdom

(72) Inventor(s)
Juha Apajalahti
Pekka Heikkinen
Janne Kerovuo
Marko Lauraeus
Andrew J Morgan
Päivi Nurminen
Osmo Siikanen

(74) continued overleaf

(51) INT CL[6]
C12N 9/16 15/55

(52) UK CL (Edition P)
C3H HB7E HC1 H650 H651 H653 H654 H655 H657 H730
C5Y YB YF YG01 YG04 YG12 YG16 Y115 Y116 Y125
Y192 Y327 Y334 Y339 Y420 Y501 Y503
U1S S1068 S1074 S1284 S1285 S1287 S1289 S1290
S1333 S1334 S1336 S1338

(56) Documents Cited
Derwent WPI Abstract Accession No.94-094823/12 & JP060038745 A Biosci.Biotech.Biochem. 1992,56(8),1266-1269 J.Bacteriology 1982,151(3),1102-1108

(58) Field of Search
UK CL (Edition O) C3H HC1
INT CL[6] C12N 9/16
ONLINE: WPI,CLAIMS,DIALOG/BIOTECH,DGENE,CAS ONLINE

(54) Phytase

(57) Phytase (preferably of microbial origin, especially isolated from Bacillus, particularly B.subtilis or B.amyloliquefaciens) has a specific activity of at least 20U/mg. protein, as determined by incubation thereof in a solution comprising 100mM Tris-HCl (pH 7.5), 1mM calcium chloride and 1.6mM sodium phytate, at 37 dgrees C for 30 minutes. The phytase may have either (i) a pH optimum of at least 6.5 by incubation thereof in a solution comprising 100mM maleic acid-Tris, 1mM CaCl2 and 1.6mM sodium phytate at 37 degrees C for 30 minutes, or (ii) a pH optimum of at least 7.0 by incubation thereof, at 37 degrees C for 30 minutes, in a solution comprising 1mM CaCl2, 1.6mM sodium phytate and either 100mM Tris-HCl or wheat bran extract.

The phytase, which may be isolated from B.subtilis BS-13 (NCIMB-40819), preferably has the structure of SEQ.ID. No.1 (not shown). Nucleic acid sequences, encoding the phytase, are disclosed. The sequences may be incoporated into a vector, which vectors are used to transform host cells, which may be prokaryotic (especially Escherichia coli, Bacillus sp., Lactobacillus sp. or Lactococcus sp.) or eukaryotic (especially fungi such as Aspergillus sp., Humicola sp., Pichia sp., Trichoderma sp. or Saccharomyces sp., and plants such as soybean, corn and rapeseed).

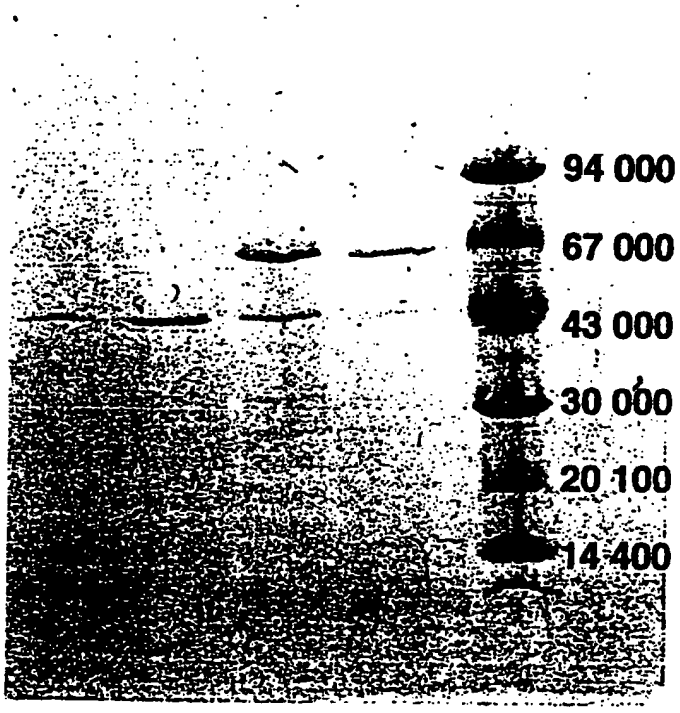
The phytase, or prokaryotic cells which express the enzyme, may be incorporated into an animal feedstuff, especially avians (paricularly poultry), ruminants (particularly bovines and ovines) and aquatic farm animals (particularly fish and shrimp). The phytase may be used either to reduce the level of phytate in animal manure, or in the production of inositol, inorganic phosphate and phosphorylated intermediates.

GB 2 316 082 A

At least one drawing originally filed was informal and the print reproduced here is taken from a later filed formal copy.

(74) Agent and/or Address for Service
Hoffmann Eitle
Sardinia House, Sardinia Street, 52 Lincoln's Inn Fields,
LONDON, WC2A 3LZ, United Kingdom

Figure 1


94 000
67 000
43 000
30 000
20 100
14 400
B13, 85% amm.sulph. precipitate
B13, 65% amm.sulph. supernatant
B13, EtOH precipitate
B13, culture supernatant
MW std

5

Figure 2

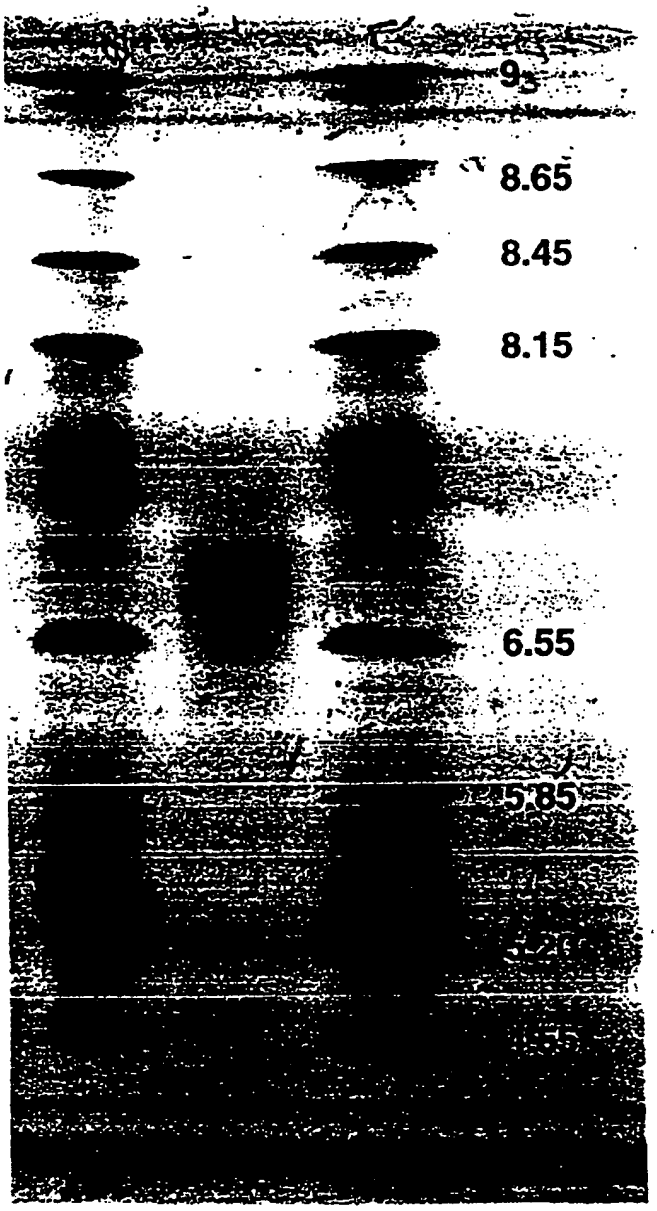
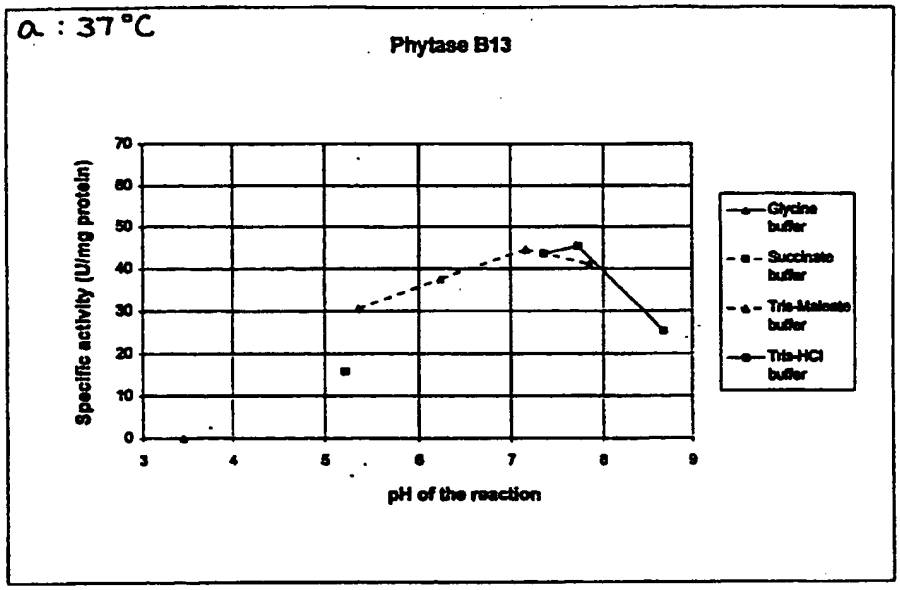
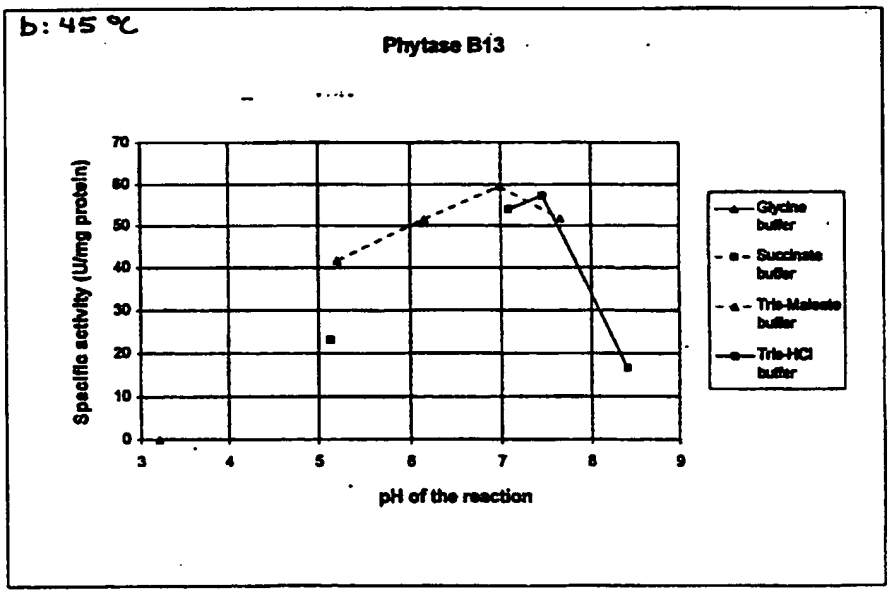
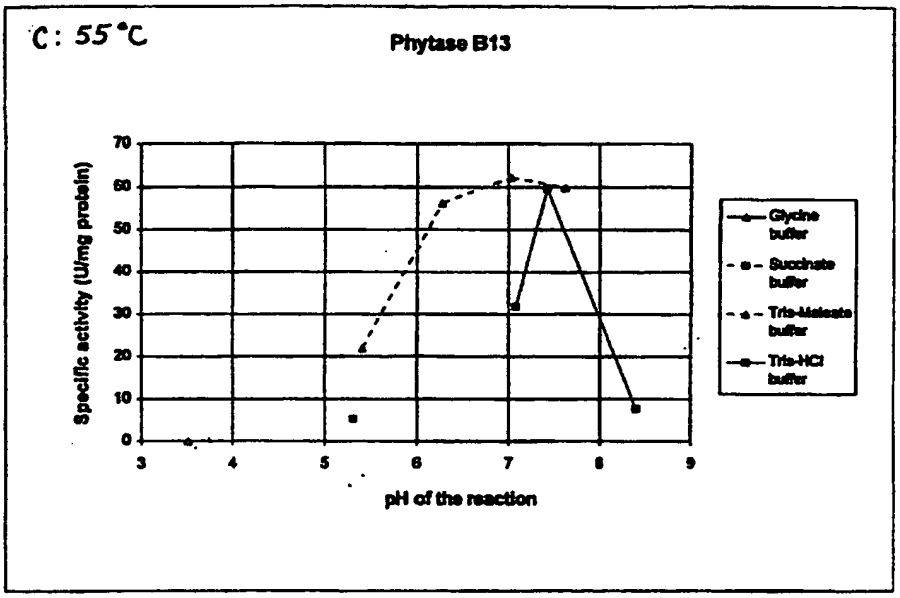


Figure 3

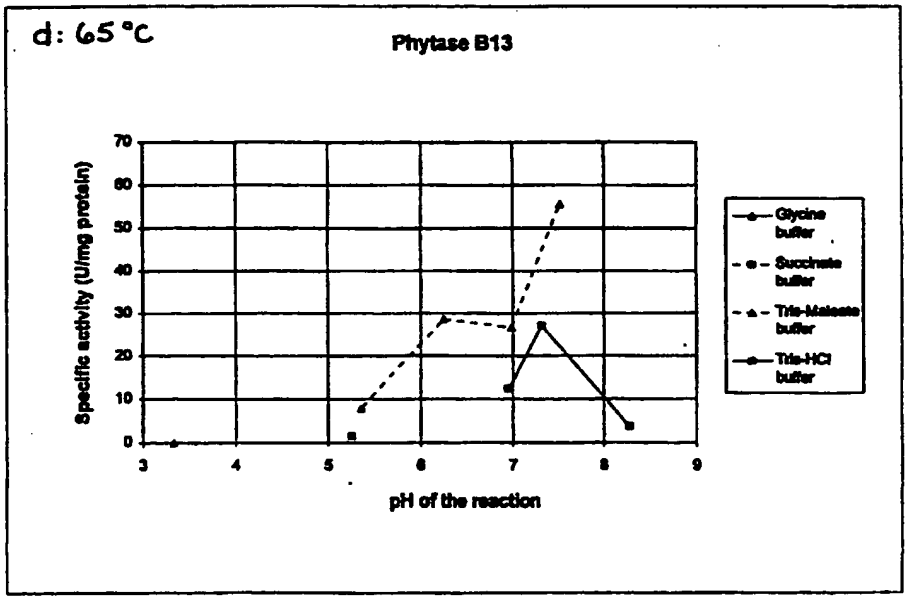

a : 37 °C
Phytase B13
Specific activity (U/mg protein)
pH of the reaction
Glycine buffer
Succinate buffer
Tris-Maleate buffer
Tris-HCl buffer


b: 45 °C
Phytase B13
Specific activity (U/mg protein)
pH of the reaction
Glycine buffer
Succinate buffer
Tris-Maleate buffer
Tris-HCl buffer

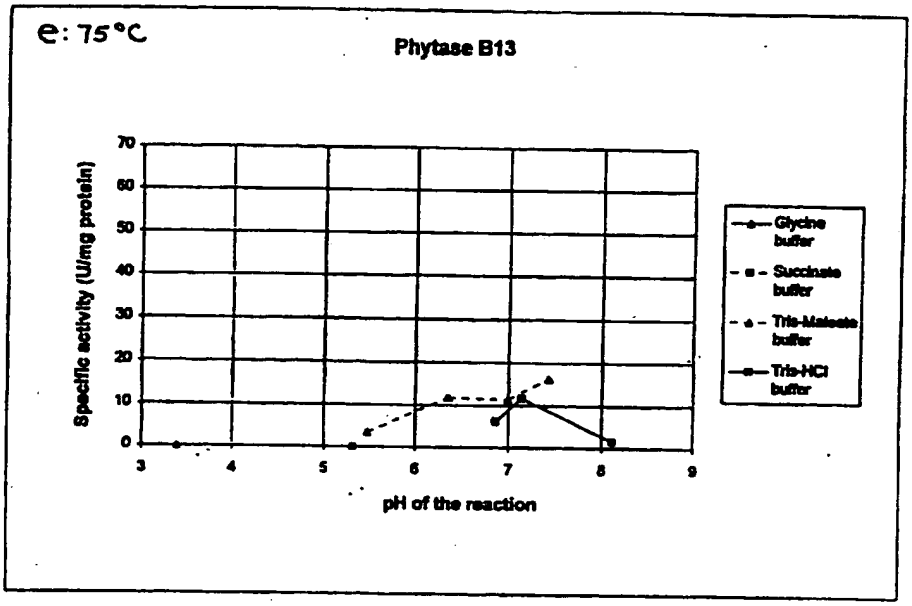
c: 55 °C


Phytase B13
Specific activity (U/mg protein)
70
60
50
40
30
20
10
0
3
4
5
6
7
8
9
pH of the reaction
Glycine buffer
Succinate buffer
Tris-Maleate buffer
Tris-HCl buffer

d: 65 °C


Phytase B13
Specific activity (U/mg protein)
70
60
50
40
30
20
10
0
3
4
5
6
7
8
9
pH of the reaction
Glycine buffer
Succinate buffer
Tris-Maleate buffer
Tris-HCl buffer

e: 75°C


Phytase B13
Specific activity (U/mg protein)
70
60
50
40
30
20
10
0
3
4
5
6
7
8
9
pH of the reaction
Glycine buffer
Succinate buffer
Tris-Maleate buffer
Tris-HCl buffer

5

Figure 4

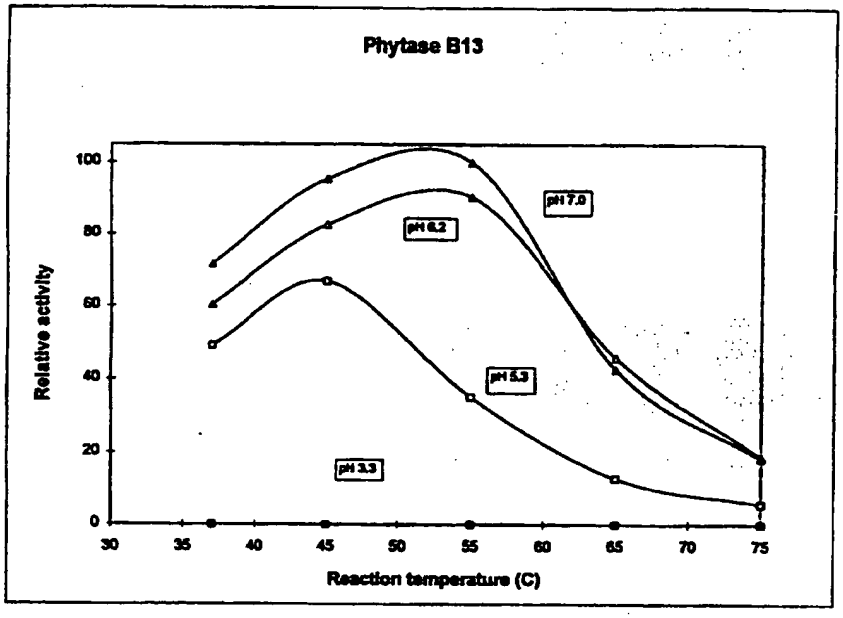
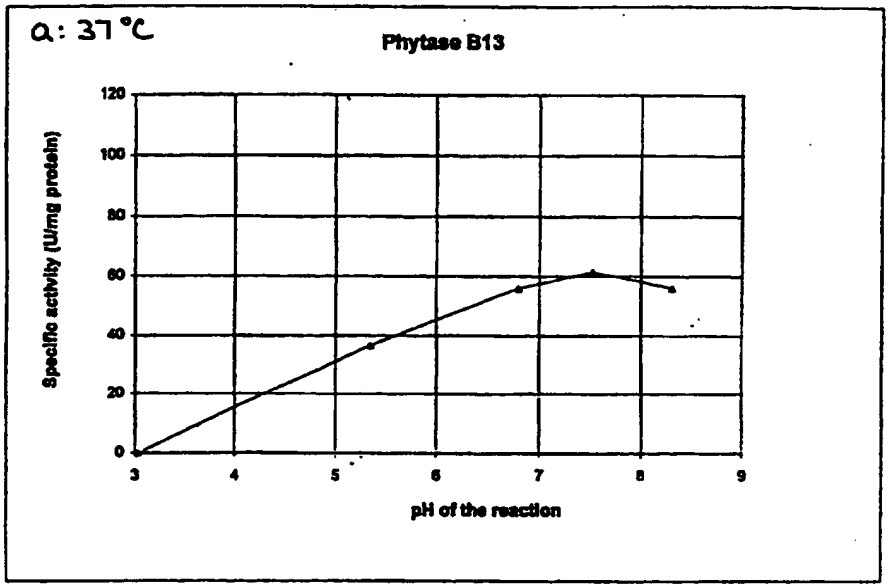
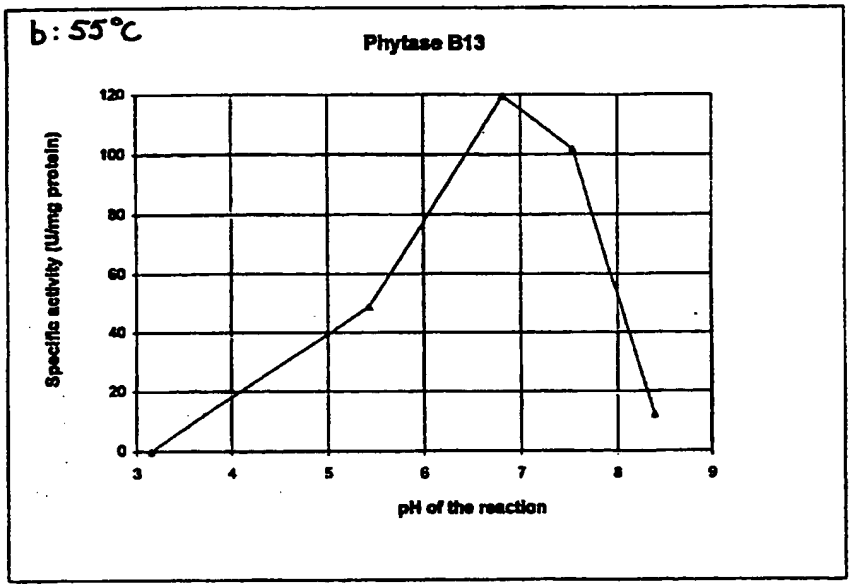

Phytase B13
Relative activity
100
80
60
40
20
0
30
35
40
45
50
55
60
65
70
75
Reaction temperature (C)
pH 7.0
pH 6.2
pH 5.3
pH 3.3

Figure 5


a: 37°C
Phytase B13
Specific activity (U/mg protein)
120
100
80
60
40
20
0
3
4
5
6
7
8
9
pH of the reaction


b: 55°C
Phytase B13
Specific activity (U/mg protein)
120
100
80
60
40
20
0
3
4
5
6
7
8
9
pH of the reaction

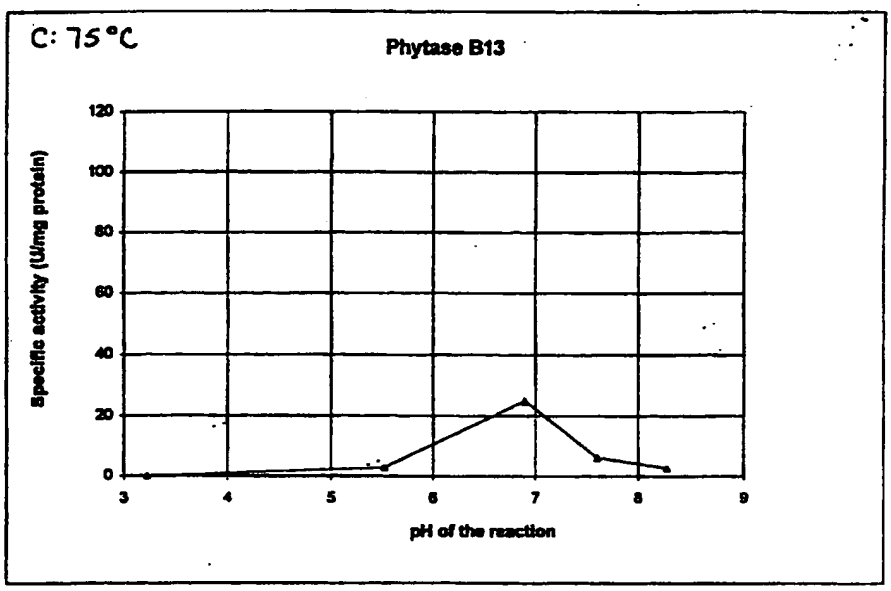

C: 75 °C
Phytase B13
Specific activity (U/mg protein)
120
100
80
60
40
20
0
3
4
5
6
7
8
9
pH of the reaction

Figure 6

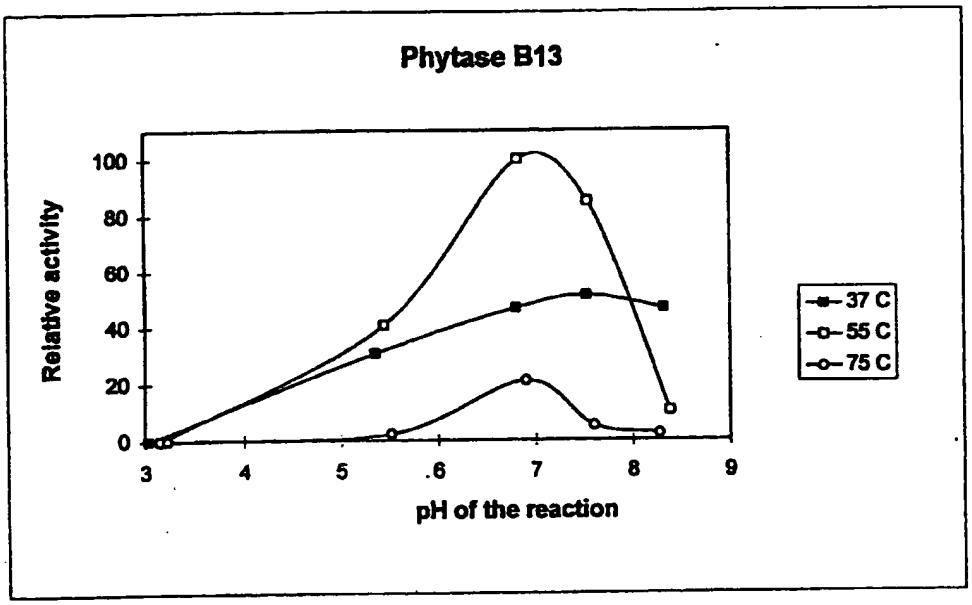
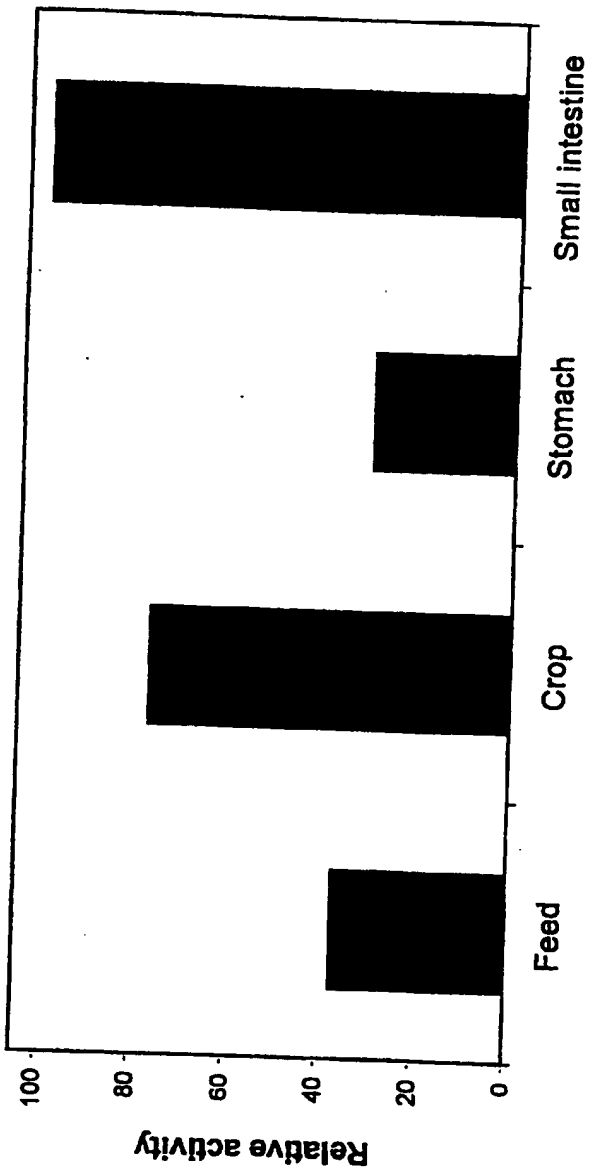

Phytase B13
Relative activity
100
80
60
40
20
0
3
4
5
.6
7
8
9
pH of the reaction
37 C
55 C
75 C

Figure 7


Small intestine
Stomach
Crop
Feed
100
80
60
40
20
0
Relative activity

5

Figure 8

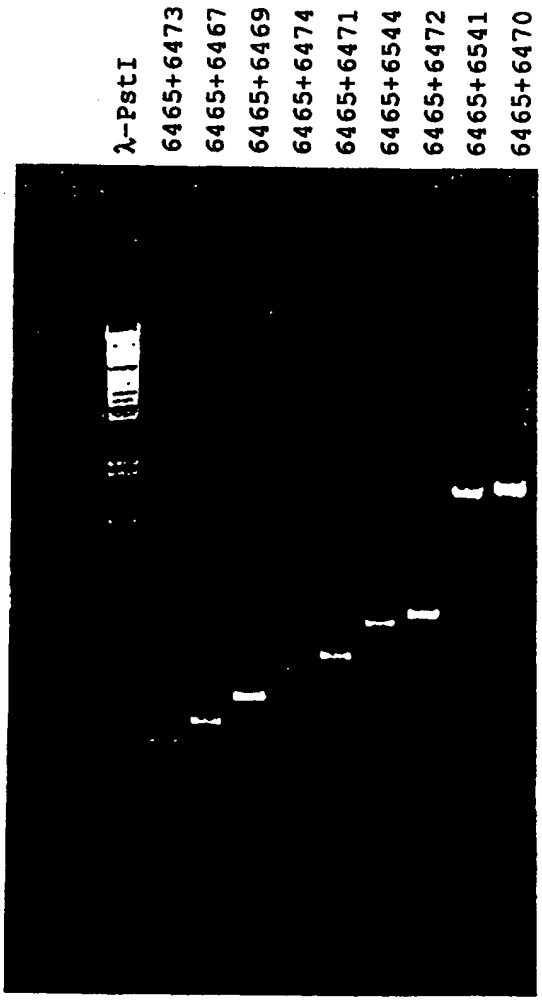
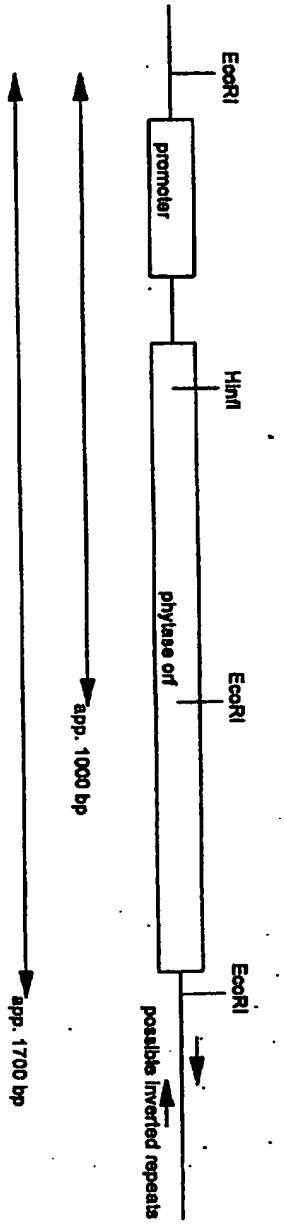

λ-PstI
6465+6473
6465+6467
6465+6469
6465+6474
6465+6471
6465+6544
6465+6472
6465+6541
6465+6470

Figure 9


EcoRI
promoter
HinfI
phytase orf
EcoRI
EcoRI
possible inverted repeats
app. 1000 bp
app. 1700 bp

Phytase, Gene Encoding Said Phytase, Method For Its Production And Use

The present invention relates to phytase, nucleic acids encoding phytase as well as methods for the production of phytase and its use.

Background of the Invention

Phosphorous is an essential element for growth. A substantial amount of the phosphorous found in many foods and animal feeds is present in the form of phosphate which is covalently bound in a molecule known as phytate (myo-inositol hexakisphosphate). Since phytate itself is poorly digested and phosphate is to a large extent absorbed in the small intestine of an animal, phosphate sequestered in phytate and not made available to an animal in the small intestine is not absorbed, passes through the digestive tract and is excreted. This leads to an increased ecological phosphorus burden to land and water. In addition, since phytate chelates several essential minerals and prevents or inhibits their absorption in the digestive tract, phytate decreases the nutritional value of food and animal feeds.

Another problem associated with poor phytate digestability is that inorganic phosphates need to be added to animal feeds, thereby increasing their costs.

Phytate is converted by enzymes known as phytases which catalyse the hydrolysis of phytate to inositol and inorganic phosphate. Phytase is found in wheat bran and plant seeds and is known to be produced by various micro-organisms including yeast, fungi and bacteria.

Among known fungal phytases, Aspergillus terreus phytase was purified to homogeneity by Yamada et al.(Agr. Biol. Chem., 32 (10) (1968), 1275-1282) and shown to have a pH optimum of pH

4.5, a temperature optimum of about 70°C at pH 4.5 and a thermal stability over a temperature range from 30 to 60 °C at pH 4.5. However, said enzyme was shown to be completely inactive at neutral pH values, particularly at pH 7.0.

In addition, the Aspergillus ficuum phytase isolated and characterised by H.J. Ullah and D.M. Gibson (Preparative Biochemistry, 17 (1) (1987), 63-91) was shown to have two pH optima, one at 2.2 and the other at 5.0-5.5, a temperature optimum of 58°C at pH 5.0 and a thermal stability up to 68°C at pH 5.0. However, as is the case with Aspergillus terreus phytase, Aspergillus ficuum phytase was shown to be inactive at pH 7.0.

DNA sequences encoding phytases from Aspergillus terreus (EP 684 313) and Aspergillus ficuum (EP 420 358) as well as Aspergillus niger var. awamori (Piddington et al., (1993) Gene, 133, 55-62) have been characterised and recombinantly expressed.

Phytases are also known from bacterial sources such as Bacillus subtilis (V.K. Powar and V. Jagannathan, (1982) J. Bacteriology, 151 (3), 1102-1108) and Bacillus subtilis (natto) (M. Shimizu, (1992) Biosci. Biotech. Biochem., 56 (8), 1266-1269 and Japanese Patent Application 6-38745).

Bacillus subtilis (natto) phytase described by Shimizu (supra) was purified to homogeneity by SDS-PAGE and was shown to have a molecular weight of between 36 and 38 kD. This enzyme was shown to have a pH optimum between pH 6.0 and 6.5 when measured in an assay solution at 37°C comprising 0.1 M maleic acid, 2 mM $CaCl_2$ and 1.6 mM sodium phytate and a pH optimum of pH 7.0 when assayed in a solution comprising 0.1 M Tris-HCl buffer, 2 mM $CaCl_2$ and 1.6 mM sodium phytate at 37°C. The temperature optimum for this phytase was shown to be 60°C and the enzyme is stable up to 50°C when incubated in the above mentioned assay solution containing Tris-HCl buffer for 15

min. The specific activity of this purified Bacillus subtilis (natto) phytase in said Tris-HCl containing solution was reported as 8.7 U/mg protein. One unit of phytase was defined as the amount of enzyme required to liberate one µmol of Pi per minute under tha assay conditions. This definition is used throughout.

Powar et al. (supra) described the isolation of a phytate specific phosphatase preparation from Bacillus subtilis which has a molecular weight of 36.5 kD. This enzyme preparation, which was purified by SDS-PAGE and found to comprise two phytase isozymes, was shown to have a pH optimum between 7.0 and 7.5 when measured in an assay solution comprising 0.1 M Tris-HCl buffer, 0.5 mM $CaCl_2$ and 0.34 mM sodium phytate at 30°C. This phytase isozyme mixture exhibited a maximum activity at a temperature of 60°C and was stable up to a temperature of 70°C. The specific activity of the purified enzyme was reported as 8.5 to 9.0 U/mg protein when measured in the above assay solution. In addition, it was reported by Powar et al (supra) that the purified isozyme mixture contained proteolytic activity which resulted in the loss of activity.

The amino acid sequence of Bacillus phytase as well as nucleic acids which encode Bacillus phytases are not known to date.

The idea of supplementing foods and animal feed with naturally occurring or recombinant phytases in order to enzymatically convert phytate to digestible phosphate during food and animal feed processing has been described. JP-A-6-38745 describes the use of purified naturally occurring Bacillus subtilis (natto) phytase for use in processing feeds and foods. In addition, EP 420 358 and EP 684 313 describe the use of Aspergillus phytase in animal feeds.

Furthermore, it has also been suggested to add phytase to animal feeds which have already been processed in order to

allow the enzymatic action of said phytases to take place in the digestive tract of the animal.

However, the above mentioned Aspergillus phytases are either inactive or lose a substantial amount of their activity at the temperature and/or pH at which foods or animal feeds are processed (generally 65 to 95°C, pH 5.5 to 7.5) and at the pH of the small intestine of monogastric animals (generally 37-41°C, pH 5.5 to 7.5).

Furthermore, the specific activity, and therefore the relative activity, of the above mentioned Bacillus phytases is very low under the above conditions.

Summary of the invention

Due to the difference in the temperatures and/or pH used during processing of foodstuffs and in the digestive tract of animals, it is desirable to have available a phytase which has a high specific activity as well as a high relative activity both at the processing temperature and/or pH of foods and animal feeds and at the temperature and/or pH in the digestive tract of animals in order to both maximise the effects of phytase during food and feed processing, during digestion within the digestive tract and to reduce the phosphorous burden to the environment resulting from digestion of phytate containing animal feedstuffs.

Moreover, a method for the production of large quantities of phytase which fulfils the above criteria is also desirable for the economic production of said foods and animal feeds.

An object of the present invention is to provide phytase with a high specific activity which is capable of functioning with a high relative activity during the processing of foods and animal feeds and/or is capable of functioning with high relative activity in the digestive tract of farmed animals.

A further object of the present invention is to provide nucleic acid molecules which encode phytase of the present invention.

A further object of the present invention is to provide methods for the production of said phytase as well as means for delivering said phytase to said animals.

Other objects of the present invention will become apparent from the following detailed specification.

Subject matter of the invention is phytase or a functional derivative thereof, characterised in that said phytase has a specific activity of at least 20 U/mg protein, wherein said specific activity is determined by incubating said phytase in a solution containing 100 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, and 1.6 mM sodium phytate at 37°C for 30 minutes. Preferably, the phytase of the present invention has a specific activity of at least 29 U/mg protein, more preferably at least 80 U/mg protein, and most preferably at least 88 U/mg protein when assayed under the above conditions.

According to a preferred embodiment, said phytase has a pH optimum of at least pH 6.5, wherein said pH optimum is determined by incubating said phytase in a solution containing 100 mM maleic acid-Tris, 1 mM $CaCl_2$, and 1.6 mM sodium phytate at 37°C for 30 minutes or a pH optimum of at least pH 7.0, wherein said pH optimum is determined by incubating said phytase in a solution containing 100 mM Tris-HCl, 1 mM $CaCl_2$, and 1.6 mM sodium phytate at 37°C for 30 minutes or by incubating said phytase in a solution containing wheat bran extract, 1 mM $CaCl_2$, and 1.6 mM sodium phytate at 37°C for 30 minutes.

It is advantageous for phytase to have a relatively high activity both during food or feed processing and in the

digestive tract of farmed animals such that the enzyme is capable of functioning well under both conditions. The activity of phytase of the present invention in feed or food during processing is preferably greater than or equal to 30%, more preferably greater than or equal to 35%, and most preferably greater than or equal to 37%, compared to the activity of said phytase in the digestive tract, preferably the crop and/or small intestine, of a farm animal.

In addition, said phytase is preferably capable of functioning in the presence of digestive enzymes found in the small intestine of animals. Enzymes which are found in the small intestine of animals include pancreatic enzymes such as trypsin, chymotrypsin and lipase.

The present invention relates to phytase with one or more of the above characteristics.

The phytase of the present invention is obtainable from a microbial source, preferably a strain of Bacillus, more preferably a Bacillus strain selected from the group comprising Bacillus subtilis and Bacillus amyloliquefaciens, and most preferably Bacillus subtilis strain B 13 deposited on August 1, 1996 at the National Collections of Industrial and Marine Bacteria, Ltd. (NCIMB) in Scotland under accession number NCIMB-40819.

In a preferred embodiment, phytase of the present invention comprises the amino acid sequence according to SEQ ID NO: 1 or a functional derivative thereof. The term "a functional derivative thereof" as it relates to phytase is used throughout the specification to indicate a derivative of phytase which has the functional characteristics of phytase of the present invention. Functional derivatives of phytase encompass naturally occurring, synthetically or recombinantly produced peptides or peptide fragments, mutants or variants which may have one or more amino acid deletions, substitutions

or additions which have the general characteristics of the phytase of the present invention.

Further subject matter of the present invention is an isolated nucleic acid or a functional derivative thereof, which encodes a phytase having one or more of the above characteristics. Preferably, said nucleic acid comprises a DNA sequence according to SEQ ID NO: 1 or a functional derivative thereof, or hybridises to a DNA sequence according to SEQ ID NO: 1 or a functional derivative thereof.

Further subject matter is an isolated nucleic acid which encodes a phytase or a functional derivative thereof, characterized in that said nucleic acid hybridises to a DNA according to SEQ ID NO: 1 and encodes a phytase having a pH optimum of greater than or equal to pH 5.0 and a specific activity of at least 10 U/mg protein as determined in a solution containing 100 mM maleic acid-Tris, 1 mM $CaCl_2$, and 1.6 mM sodium phytate at 37°C for 30 minutes.

Said nucleic acid is preferably a DNA molecule. The term "a functional derivative thereof" as it relates to nucleic acids encoding phytase is used throughout the specification to indicate a derivative of a nucleic acid which has the functional characteristics of a nucleic acid which encodes phytase. Functional derivatives of a nucleic acid which encode phytase of the present invention encompass naturally occurring, synthetically or recombinantly produced nucleic acids or fragments, mutants or variants thereof which may have one or more nucleic acid deletions, substitutions or additions and encode phytase characteristic of the present invention. Variants of nucleic acid encoding phytase according to the invention include alleles and variants based on the degeneracy of the genetic code known in the art. Mutants of nucleic acid encoding phytase according to the invention include mutants produced via site-directed mutagenesis techniques (see for example, Botstein, D. and Shortle, D., 1985, Science 229:

1193-1201 and Myers, R.M., Lerman, L.S., and Maniatis, T., 1985, Science 229: 242-247), error-prone PCR (see for example, Leung, D.W., Chen, E., and Goeddel, D.V., 1989, Technique 1: 11-15; Eckert, K.A. and Kunkel, T.A., 1991, PCR Methods Applic. 1: 17-24; and Cadwell, R.C. and Joyce, G.F., 1992, PCR Methods Applic. 2: 28-33) and/or chemical-induced mutagenesis techniques known in the art (see for example, Elander, R.P 'Microbial screening, Selection and Strain Improvement' in Basic Biotechnology, J. Bu'lock and B. Kristiansen Eds., Academic Press, New York, 1987, 217).

Subject matter of the present invention is also a method for the production of a nucleic acid of the invention, characterised in that a probe comprising a nucleic acid as described above is hybridised under standard conditions to a sample suspected of containing said nucleic acid and said nucleic acid is recovered. Standard techniques employing said probe for hybridisation include Southern blotting (see for example, Sambrook et al., Molecular Cloning, a Laboratory Manual, 2nd. Edition, Cold Spring Harbor Laboratory Press, 1989), PCR and RT-PCR(see for example, PCR Protocols: A Guide to Methods and Applications, Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. Eds., Academic Press New York, 1990). Standard conditions for hybridization are preferably 6 x SSC, 0.5% SDS, 50°C overnight or functional equivalents thereof for Southern blotting and for PCR: 5 mM Mg^{2+}, Taq enzyme, premelting, 94°C for 2 min and 30 cycles of melting at 92°C for 20 sec, annealing at 50°C for 30 sec and extension at 72 °C for 1 min, or functional equivalents thereof.

Subject matter of the present invention is also a vector comprising a DNA molecule of the present invention. Preferably, said vector is characterised in that said DNA molecule is functionally linked to regulatory sequences capable of expressing phytase from said DNA sequence. Preferably, said DNA molecule comprises a leader sequence capable of providing for the secretion of said phytase. Said

regulatory sequences can comprise prokaryotic or eukaryotic regulatory sequences.

Depending on whether the phytase of the invention is expressed intracellularly or is secreted, a DNA sequence or vector of the invention can be engineered such that the mature form of the phytase of the invention is expressed with or without a natural phytase signal sequence or a signal sequence which functions in Bacillus, other prokaryotes or eukaryotes. Expression can also be achieved by either removing or partially removing said signal sequence.

Subject matter of the present invention is also a prokaryotic host cell transformed by a nucleic acid or vector as described above. Preferably said host cell is selected from the group comprising E. coli, Bacillus sp., Lactobacillus and Lactococcus.

Subject matter of the present invention is also a eukaryotic host cell transformed by a nucleic acid or vector as described above. Preferably said host cell is selected from the group comprising Aspergillus sp., Humicola sp., Pichia sp., Trichoderma sp. Saccharomyces sp. and plants such as soybean, maize and rapeseed.

Subject matter of the present invention is also a method for the recombinant production of phytase, characterised in that a prokaryotic or eukaryotic host cell as described above is cultured under suitable conditions and said phytase is recovered.

A preferred embodiment of the phytase of the present invention is a phytase obtainable according to the above method.

Further subject matter of the present invention is the use of bacterial cells or spores capable of producing phytase according to the invention as a probiotic or direct fed

microbial product. Preferred embodiments for said uses are phytase-producing Bacillus sp. and Lactobacillus sp. of the invention.

Further subject matter of the invention is also a use of phytase according to the present invention in food or animal feed.

Further subject matter is food or animal feed comprising phytase according to the invention. Preferably, said food or animal feed comprises phytase as an additive which is active in the digestive tract, preferably the crop and/or small intestine, of said animal, wherein said animal is preferably selected from the group comprising avians including poultry, ruminants including bovine and sheep, pig, and aquatic farm animals including fish and shrimp. Said additive is also preferably active in food or feed processing.

Further subject matter is food or animal feed comprising prokaryotic cells or spores capable of expressing phytase according to the present invention.

Subject matter of the present invention is also a method for the production of a food or animal feed, characterised in that phytase according to the invention is mixed with said food or animal feed. Said phytase is added as a dry product before processing or as a liquid before or after processing. If a dry powder is used, the enzyme would be diluted as a liquid onto a dry carrier such as milled grain.

Subject matter of the present invention is also a method for the production of a food or animal feed, characterised in that prokaryotic cells and/or spores capable of expressing phytase according to the inveniton are added to said food or animal feed.

Subject matter of the present invention is also a use of phytase according to the invention with or without accessory phosphatases in the production of inositol and inorganic phosphate.

Further subject matter of the present invention is a method for the reduction of levels of phosphorous in animal manure, characterised in that an animal is fed an animal feed according to the invention in an amount effective in converting phytate contained in said animal feed.

Definitions

The term "phytase" is defined throughout the specification as a protein or polypeptide which is capable of catalysing the hydrolysis of phytate and releasing inorganic phosphate.

Specific activity of phytase is defined throughout specification as the number of units (U)/ mg protein of a solution comprising phytase, wherein said phytase is detectable as a single band by SDS-PAGE. One unit is the amount of enzyme required to liberate one µmol of Pi per minute when said enzyme is incubated in a solution containing 100 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, and 1.6 mM sodium phytate at 37°C for 30 minutes.

Relative activity of phytase is defined throughout the specification as the activity of the enzyme at a given temperature and/or pH compared to the activity of the enzyme at the optimal temperature and/or pH of said enzyme.

Brief description of the drawings

Figure 1: SDS-PAGE gel of phytase purification(procedure);

Figure 2: Isoelectric focusing gel of purified phytase;

Figure 3: Effect of pH on the activity of phytase at different temperatures;

Figure 4: Effect of pH on the temperature activity profile of phytase in defined buffers;

Figure 5: Effect of pH on the activity of phytase in wheat bran extract at different temperatures;

Figure 6: Effect of pH on the temperature activity profile of phytase in wheat bran extract;

Figure 7: Relative activity of phytase under pH and temperature corresponding to feed processing and digestion processes;

Figure 8: Results of PCR amplification of gene encoding B. subtilis phytase using primers derived from amino acid sequence;

Figure 9: Structure of B. subtilis phytase gene; and

Detailed description of the invention

The invention is more closely illustrated by the following examples.

Example 1

Bacillus subtilis B 13 deposited at the National Collections of Industrial and Marine Bacteria, Ltd. (NCIMB) in Scotland under accession number NCIMB-40819 was used throughout the study.

Media

Luria medium, containing 5 g of yeast extract, 10 g of tryptone and 10 g of NaCl per litre, was used to grow the inoculum for the production of phytase.

Wheat bran extract was used as the enzyme production medium and it was prepared as follows. 100 grams of wheat bran was extracted with 1000 ml of water by autoclaving at 121°C for 60 minutes. The extract was filtered through six layers of cheesecloth and then the volume of the extract was adjusted to one litre by addition of water. This extract was supplemented with: $(NH_4)_2SO_4$ 0.4 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, casitone 10 g, KH_2PO_4 0.5 g and K_2HPO_4 0.4 g. The final pH of the extract was 6.5. The extract base was autoclaved at 121°C for 15 minutes. Prior to inoculation, 5% $CaCl_2$ (filter sterilised) was added to the final concentration of 0.2%.

Production of enzyme

Inoculum was grown up from the frozen stock in Luria medium supplemented with 0.2% $CaCl_2$. The initial inoculum was grown for 24 hours at 30°C in a rotatory shaker. The cultivation was scaled up using successive 10% inoculations in wheat bran

medium. For enzyme production the 5 litre batch was grown in wheat bran medium at 30°C for 91 hours with vigorous shaking.

Protein assay

Protein concentrations were determined by Bio-Rad Protein Microassay Procedure according to the recommendations of the manufacturer by using Bovine Serum Albumin as a standard.

Purification of phytase

All purification steps were carried out at 0 - 4°C unless otherwise stated. Bacteria were pelleted by centrifugation at 7000 x g for 30 minutes. The volume of the collected supernatant was determined and $CaCl_2$ added to a final concentration of 1 mM. The enzyme was precipitated by adding three volumes of cold (-20°C) ethanol, which was added with constant stirring to the supernatant. Stirring was continued for 45 minutes and the precipitation was carried out overnight. The precipitate was collected by centrifugation at 1800 x g for 20 minutes. The collected precipitate was washed once with cold (-20°C) ethanol and once with cold (-20°C) acetone. Excess acetone was evaporated from the precipitate under nitrogen gas flow and then the drying was completed by lyophilisation.

The dried precipitate was dissolved in 300 ml of 100 mM Tris-HCl, pH 7.5, supplemented with 1 mM $CaCl_2$. Ammonium sulphate was added slowly to the solution under constant stirring until 65% saturation was reached. The solution was incubated at 4 °C overnight, cleared by centrifugation at 9000 x g for 60 minutes at 4°C and then ammonium sulphate added until 85% saturation was reached. The solution was again incubated over night at 4 °C. Precipitate was collected by centrifugation as before and then dissolved in 100 mM Tris-HCl, pH 7.5, supplemented with 1 mM $CaCl_2$. Aliquots of enzyme preparation were stored at -20°C. When used for experiments the enzyme

preparations were gel filtered to a desired defined buffer by using PD-10 (Pharmacia) gel filtration columns. The purification scheme of phytase is shown in Table 1.

Table 1: Specific activity of purified phytase

Enzyme sample	volume (ml)	Protein conc. (mg/ml)	specific activity (U/mg)	total activity (U)	recovery (%)	purification factor
culture supernatant	5000	0.3	8	10270	100	1.00
rediss. EtOH precipitate	305	2.1	15	9528	93	1.91
supernatant 65% $(NH_4)_2SO_4$	330	0.2	88	5720	56	11.19
rediss. pellet 85% $(NH_4)_2SO_4$	20	3.8	29	2231	22	3.69

Estimation of molecular weight and isoelectric point

The molecular weight of phytase as purified above was estimated in Pharmacia Phast electrophoresis equipment by using SDS 8-25% gradient polyacrylamide gel electrophoresis (PhastGel ® SDS-page) and the Pharmacia Low Molecular Weight Electrophoresis Calibration Kit as a standard according to recommendations by the manufacturer. The isoelectric point was determined with the same system using PhastGel IEF 3-9 isoelectric focusing gel and the Pharmacia IEF Calibration Kit as a standard.

Molecular weight of the B 13 phytase was 43,000 as determined by SDS-PAGE (Figure 1). Isoelectric pH of the B 13 phytase was 6.5 (Figure 2).

Substrate specificity

Substrate specificity of the phytase (in 0.1 M Tris-HCl, pH 7.5) was determined by using the standard activity assay of each enzyme. Besides phytic acid, β-glycerophosphate, D-glucose-6-phosphate, p-nitrophenylphosphate, ATP, ADP, AMP, fructose, 1,6-diphosphate, 3-phosphoglyceric acid, bis-(p-nitrophenyl)phosphate and α,β-methyleneadenosine-5'-diphosphate were used as alternative substrates. The results of the analysis of substrate specificity are shown in Table 2.

Table 2: Substrate specificity of phytase

Substrate	Relative activity of phytase
phytic acid	100
β-glycerophosphate	0
D-glucose-6-phosphate	0
p-nitrophenylphosphate	0
ATP	50
ADP	75
AMP	0
fructose-1,6-phosphate	0
3-phosphoglyceric acid	0
methyleneadenosine-5'-diphosphate	0
bis-(p-nitrophenyl)phosphate	0

Enzyme assay

Unless otherwise stated, the activity of phytase was measured by incubating 150 µl enzyme preparation with 600 µl of 2 mM sodium phytate in 100 mM Tris-HCl buffer pH 7.5, supplemented with 1 mM $CaCl_2$ for 30 minutes at 37°C. After incubation the reaction was stopped by adding 750 µl of 5% trichloroacetic acid. Phosphate released was measured against phosphate

standard spectrophotometrically at 700 nm after adding 1500 µl of the colour reagent (4 volumes of 1.5% ammonium molybdate in 5.5% sulphuric acid and 1 volume of 2.7% ferrous sulphate; Shimizu, M., 1992; Biosci. Biotech. Biochem., 56:1266-1269). One unit of enzyme activity was defined as the amount of enzyme required to liberate one µmol Pi per min under assay conditions. The specific activity was expressed in units of enzyme activity per mg protein. The characteristics of the phytase purified in the above manner are summarised in Table 3.

Table 3: Characteristics of phytase

Property	phytase
Molecular weight	43,000
Isoelectric point	6.5
Optimum pH at 37°C	7.5
Optimum temperature	55°C (pH 7.1)

pH and temperature activity profiles

Temperature and pH activity profiles of phytase were analysed in defined buffers and in wheat bran extract. The enzyme concentrations used in the assays gave linear orthophosphate release for the 30 minute incubation period under optimum conditions at 37°C.

Defined buffers used were 100 mM Glycine pH 3.0, 100 mM Succinate pH 5.0, 100 mM Tris-maleate pH 5.0, 6.0, 7.0 and 8.0, 100 mM Tris-HCl pH 7.5, 8 and 9. All buffers were supplemented with 2 mM sodium phytate and 1 mM $CaCl_2$. Enzyme assays were performed in these buffers at five different temperatures (37, 45, 55, 65 and 75°C). 600 µl of a buffer was temperated at the relevant temperature and the enzyme reaction was started by adding 150 µl of an enzyme preparation. Reactions were stopped after 30 minutes incubation and liberated inorganic orthophosphate was

measured as earlier described. Enzyme assays were run in duplicates. The true pH in the reaction mixture was measured in the beginning and at the end of each assay. Protein concentrations were measured as described earlier and the specific activities of enzymes were calculated at various pH and temperature.

Wheat bran extract was prepared by dissolving 50 g wheat bran in 500 ml of distilled water followed by autoclaving at 121°C for 60 minutes. The extract was filtered through cheese cloth, volume adjusted to 500 ml with distilled water and then the extract was centrifuged at 15,000 rpm for 15 minutes and the supernatant collected. The aliquots of the supernatant were adjusted to pH 3.0, 5.5, 7.0, 8.0 and 9.0, diluted 1:10 in distilled water and supplemented with 2 mM sodium phytate and 1 mM $CaCl_2$. 600 µl of a pH adjusted wheat bran extract was temperated to desired temperature (37, 55 and 75°C) and the enzyme reactions were started by adding 150 µl of enzyme preparation. Reactions were stopped after 30 minutes incubation and liberated inorganic orthophosphate was measured as described above. Enzyme assays were assayed in duplicates. The true pH of each reaction mixture was measured in the beginning and at the end of the enzyme assay.

Effect of pH on the phytase activity

Relative activity of phytase was determined over a pH ranging from 3.0 to 8.5 using both defined buffers and pH adjusted wheat bran extract. It was obvious that not only the pH of the buffer, but also acid composition of the buffer affected relative phytase activity. To cover the pH range, four different defined buffers or wheat bran extract, the pH of which was adjusted by HCl or NaOH addition, were used. Since enzyme addition affected pH of the reaction mixture, the true pH of each assay mixture was measured in the beginning and in the end of the 30 minute incubation. During the reaction the changes of pH were insignificant.

True reaction pH was used in the determination of pH activity profiles.

Figures 3a to 3e show the pH activity profiles of B 13 phytase in defined buffers at five different temperatures between 37 and 75°C. Irrespective of the reaction temperature, phytase showed highest phytase activity at pH 7.5.

Animal compound feed typically has a pH ranging from pH 5.5 to 7.5.

Temperature optimum of phytase was 55°C. The effect of pH on the temperature activity profile of phytase in the above defined buffers is shown in Figure 4.

Wheat bran extract is likely to provide an environment that is closer to feed and animal digesta than any of the defined buffers. We determined the pH activity profiles of the phytases at 37, 55 and 75°C. Activity of the enzyme in wheat bran extract doubled as compared to its activity in defined buffers (Figures 5a to 5c). The profiles did not differ from those found in the defined buffers (Figure 6).

Figure 7 illustrates the relative activity of the two phytases under pH and temperature conditions relevant to feed manufacturing and the digestive process of the broiler chicken. The data for this presentation has been taken from the experiment described above (Figures 5a to 5c).

Example 2: Cloning of the gene encoding phytase

N-terminal sequencing

The N-terminal sequence of B. subtilis B 13 phytase purified by SDS-PAGE was sequenced with a Perkin-Elmer Procice Sequencing System using Edman degradation. A twenty five amino

acid long N-terminal sequence was obtained. To obtain more information about the amino acid sequence, the purified phytase was digested with lysC enzyme to obtain internal peptides and the digest was purified with RP-HPLC. LysC digestion was also performed to alcylated phytase following RP-HPLC purification. Non-alcylated RP-HPLC purified phytase peptides were sequenced with same system. Alcylation of phytase was done to determine whether possible sulphur bridges were present. There was no difference between alcylated and non-alcylated phytase lysC digestion RP-HPLC chromathograms showing that there were no sulphur bridges in the phytase.

Nineteen purified peptides were sequenced giving fourteen peptides which were different from each other (5 to 32 amino acids) and a total of 227 amino acids. All peptide sequences are shown in Table 4, including the sequence corresponding to the N-terminus of phytase. The molecular weight of the peptides was measured using mass spectrometer and compared with calculated molecular weights.

Table 4: Peptides obtained by N-terminal amino acid sequencing

MW (det.)	MW (calc.)	amino acid sequence
		LSDPYHFTVNAAAKTEPVDTAGDAA *
		LSDPYHFTVNAAAKTEPVDTAGDAADDPAILD
932	932.1	YYAMVTGK
1271.4	1271.3	EGEFEQYELK
1050.3	1050.2	MLHSYNTGK
798.9	798.9	IVPWER
2951.2	2948.4	IVPWERIADQIGFRPLANEQVDPRK
3467		NGTLQSMTDPDHPIATAINEVYGFTLWHSQ
5450.2		YVADFRITDGPETDGTSDDDGII
775.7	775.8	LTDRSGK
1317.9	1317.4	VDIAAASNRSEGK
2167.4	2167.4	IADQIGFRPLANEQVDPRK
720.7	720.8	ANQNFK
619.6	619.7	VRAFK
		LNNVDIRYDFP
1779.4	1778	LNNVDIRYDFPLNGK
1236.3	1236.4	NTIEIYAIDGK
1137.4	1137.3	SGLVVYSLDGK
		FSAEPDGGSNGTVIDRADGRHL

* N-terminal sequence

Identification of phytase coding sequences by PCR

On the basis of these peptide sequences, primers for PCR were designed (see Table 5). All PCR were performed using a PTC-255 DNA Engine and Perkin-Elmer Taq polymerase.

Table 5: PCR primers giving only one fragment each under optimal conditions

number	oligonucleotide sequence
6465	TCIGATCCITATCATTTTACIGT
6467	AG(C/A)GGAAAATCATAIC(C/T)(G/A)ATATC
6469	CITCIGAIC(G/T)(G/A)TTIGAIGCIGC
6470	TGATCIGC(G/A)ATIC(G/T)TTCCCA
6471	GC(G/A)AT(C/A)GGATGATC(C/A)GGATC
6472	TTCATA(C/T)TGTTCAAATTCICC
6473	TTICCIGT(G/A)TTATAIGAATGIA(G/A)CAT
6474	CCATC(G/A)ATIGCATA(G/A)ATTTC
6541	TTTAAA(G/A)TT(C/T)TG(G/A)TTIGC
6544	TTTICCIGTIACCATIGC

N = A, T, G or C; I = inosine;

PCR was performed with these primers using B. subtilis B 13 DNA isolated according to Sambrook el al. (supra) as the template at different annealing temperatures (45, 50, 55 and 60 C) and at different magnesium concentrations (1.25, 2.5, 5 and 10 mM) to optimize PCR conditions. The following PCR protocol was chosen: 94°C pre-melting for 2 min. before 30 cycles of 92°C melting for 20 sec., 50°C annealing for 30 sec., 72°C extension for 60 sec. in 5 mM magnesium concentration. The primers given in Table 5 amplified only one fragment each under optimal conditions. These amplified PCR fragments are shown in Figure 8.

The longest PCR fragment (amplified with primers 6465 and 6470) was cloned to pCR 2.1 vector (Invitrogen Corp., Inc., San Diego, USA) and sequenced using Sanger Dideoxy method. This resulted in determination of the partial DNA sequence (exact length 989 bp) of phytase of the present invention.

Restriction enzyme analysis of PCR products of phytase gene

To verify that these PCR fragments were phytase fragments, restriction enzyme Hinf I which cleaves the shortest PCR fragment into two approximately 100 bp long fragments was used. These fragments cut with Hinf I gave the same sized fragment from the N-terminal end. PCR fragments were also cut with EcoRI; two of the longest phytase PCR fragments cut with EcoR I which confirms the scheme presented in the Figure 9.

Southern blot analysis of phytase of the phytase gene

Genomic DNA was isolated from *B.* subtilis B 13, as described in Sambrook et. al. (supra, 1989). Restriction enzymes used were those of Boehringer-Mannheim. B. subtilis B 13 DNA was partially digested with EcoRI and the fragments were separated on agarose gel. Separated fragments were Southern-Blotted to nylon membrane. Nylon membrane was Southern-Hybridized with 32P-labelled N-terminal oligonucleotide probe, GA(C/T)CC(G/A/T)TA(C/T)CA(C/T)TT(C/T)AC(G/A/T)GTNAA(C/T)GC (G/A/T)GC(G/A/T)GC(G/A/T)GAAAC, in order to determine the approximate size of the fragment containing the putative phytase gene. Southern-Hybridisation showed two bands of approximately 1700 bp and 1000 bp consistant with the structure of the gene given in Figure 9.

Screening of a B. subtilis B 13 genomic library

Partially EcoR I digested genomic B. subtilis B 13 DNA was cloned into Lambda Zap II using a Stratagene Lambda Zap II/EcoRI/CIAP Cloning kit according to the recommendations of the manufacturer. Lambda Zap II library was screened with Boehringer-Mannheim EasyToHyb hybridisation kit according the recommendations provided by the manufacturer using the above mentioned longest PCR fragment (989 bp) labeled with digoxigenin as the hybridisation probe.

XL-1 Blue MRF' host cells were infected with 100 000 pfu's of lambda Zap II *B.* subtilis *B* 13 genomic library phages. Infected cells were plated with TOP agarose on LB agar plates. Formed plaques were transferred to nylon membranes and screened with the 989 bp digoxigenin labeled hybridisation probe. Several intense positive clones were found with practically no backround. These positive plaques were cored and used in a second round of hybridisation. Positive plaques remained positive in a second round of hybridisation and were cored and excised with helper phage to obtain pBluescript SK(-) phagemid. Obtained phagemids were transformed to E. coli host cells and DNA from minipreps were used in analysis of insert DNA and DNA sequencing.

Determination of the DNA sequence of the gene encoding phytase

The DNA sequence encoding for phytase as well as the deduced amino acid sequence are shown in SEQ ID NO: 1. The molecular weight of phytase as deduced from the amino acid sequence in SEQ ID NO: 1 is ca. 41,900 daltons for the pre-protein and ca. 39,000 for the mature protein (i.e. without the signal sequence). This is in agreement with the molecular weight of phytase as determined from SDS-PAGE (Figure 1).

The N- terminus of the mature protein corresponds to amino acid number 30 (Leu-30) of SEQ. ID. NO: 1.

Example 3: Expression of recombinant phytase in E. coli

DNA coding for the mature protein was amplified by PCR using primers which also contained restriction sites for cloning into vectors pQE-30 and pQE-60 (Qiagen, Chatsworth, CA, USA). The 5' primer in each case encoded a Mfe I site (compatible with Eco RI) followed by a ribosome binding site and the amino terminus of the mature protein. The 3' primer for the pQE-30 construct hybridized downstream of the stop codon of the native protein followed by a Sal I site for cloning. The

resulting PCR product was cloned into pQE-30 digested with Eco RI/Sal I. This construct should produce the same protein as the mature native product with an additional methionine residue on the amino terminus.

5' primer for both pQE-30 and pQE-60 constructs:

GTTTCTCAATTGAAGGAGGAATTTAAATGCTGTCCGATCCTTATCATTTTAC
Mfe I RBS MetLeuSerAspProTyrHisPhe

3' primer for pQE-30 construct:

AATAAGTCGACGTACGACCGGATTCCGGCTGTGCT
Sal I

The 3' primer used for the pQE-60 construct encoded the C-terminus of the protein (without stop codon) followed by a Bgl II cloning site. The vector sequence provides the nucleotides encoding a histidine tag to facilitate purification of the expressed protein. The PCR product was cloned into pQE-60 digested with Eco RI/Bgl II. The enzyme expressed from this construct can be purified from the cell lysate using Ni-NTA resin according to the manufacturer's instructions (Qiagen).

3' primer for pQE-60 construct:

AATAAAGATCTTTTTCCGCTTCTGTCGGTCAGTT
Bgl II

Said constructs were then transformed into the expression host M15/pREP4 cell line (Quiagen). The M15/pREP4 cell line was made competent and transformed using standard procedures (Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harboe, New York, 1989). This cell line contains a plasmid (pREP4) which constitutively expresses the lac repressor protein. This allows strong repression of the

expression constructs in pQE-30 and pQE-60 which have two lac repressor recognition sequences upstream of the open reading frame. The vectors use the phage T5 promoter which is efficiently recognized by the E. coli RNA polymerase. These constructs were grown overnight in LB medium supplimented with ampicillin, methicillin and kanamycin at 37°C. The overnight cultures were diluted 1:30 in fresh media and grown to OD_{600} 0.8 at which point they were induced with 1.5 mM IPTG. After three additional hours of growth, the cells were havested, washed, and lysed by sonication. The lysates were cleared of debris by centrifugation. Aliquots of cleared lysates were also assayed for enzyme activity. The assays were performed in reaction buffer (100 mM Tris-100 mM maleate, pH 7, 1 mM $CaCl_2$ and 2 mM sodium phytate) at 42°C for 30 minutes. The results are presented in Table 6.

Table 6

construct	assay	background	difference
pQE	0.044	0.007	0.037
pQE-30	0.259	0.002	0.257
pQE-60	1.160	0.004	1.156

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Finnfeeds International, Ltd.

(B) STREET: P.O. Box 777

(C) CITY: Marlborough

(D) STATE: Wiltshire

(E) COUNTRY: United Kingdom

(F) POSTAL CODE (ZIP): SN8 1XN

(ii) TITLE OF INVENTION: Phytase, gene encoding said phytase, method for its production and use

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1290 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Bacillus subtilis

(B) STRAIN: B13

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:91..1239

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
CACATTTGAC AATTTTCACA AAAACTTAAC ACTGACAATC ATGTATATAT GTTACAATTG      60

AAGTGCACGT TCATAAAAGG AGGAAGTAAA ATG AAT CAT TCA AAA ACA CTT TTG      114
                                 Met Asn His Ser Lys Thr Leu Leu
                                   1               5

TTA ACC GCG GCG GCC GGA CTG ATG CTC ACA TGC GGT GCG GTG TCT TCC      162
Leu Thr Ala Ala Ala Gly Leu Met Leu Thr Cys Gly Ala Val Ser Ser
     10                  15                  20

CAG GCA AAG CAT AAG CTG TCC GAT CCT TAT CAT TTT ACC GTG AAT GCA      210
Gln Ala Lys His Lys Leu Ser Asp Pro Tyr His Phe Thr Val Asn Ala
 25                  30                  35                  40

GCG GCG GAA ACG GAA CCG GTT GAT ACG GCC GGT GAC GCG GCT GAT GAT      258
Ala Ala Glu Thr Glu Pro Val Asp Thr Ala Gly Asp Ala Ala Asp Asp
                 45                  50                  55
```

```
CCT GCG ATT TGG CTG GAC CCC AAG ACT CCT CAG AAC AGC AAA TTG ATT      306
Pro Ala Ile Trp Leu Asp Pro Lys Thr Pro Gln Asn Ser Lys Leu Ile
            60                  65                  70

ACG ACC AAT AAA AAA TCA GGT TTA GTC GTT TAC AGC CTT GAT GGT AAG      354
Thr Thr Asn Lys Lys Ser Gly Leu Val Val Tyr Ser Leu Asp Gly Lys
        75                  80                  85

ATG CTT CAT TCC TAT AAT ACC GGG AAG CTG AAC AAT GTC GAT ATC CGT      402
Met Leu His Ser Tyr Asn Thr Gly Lys Leu Asn Asn Val Asp Ile Arg
    90                  95                  100

TAT GAT TTT CCG TTG AAC GGC AAA AAA GTC GAT ATC GCG GCA GCA TCC      450
Tyr Asp Phe Pro Leu Asn Gly Lys Lys Val Asp Ile Ala Ala Ala Ser
105                 110                 115                 120

AAT CGG TCT GAA GGA AAA AAT ACC ATT GAG ATT TAC GCT ATT GAT GGA      498
Asn Arg Ser Glu Gly Lys Asn Thr Ile Glu Ile Tyr Ala Ile Asp Gly
                125                 130                 135

AAA AAC GGC ACA TTA CAA AGC ATG ACA GAT CCA GAC CAT CCG ATT GCA      546
Lys Asn Gly Thr Leu Gln Ser Met Thr Asp Pro Asp His Pro Ile Ala
            140                 145                 150

ACA GCA ATT AAT GAG GTA TAC GGT TTT ACC TTA TAC CAC AGT CAA AAA      594
Thr Ala Ile Asn Glu Val Tyr Gly Phe Thr Leu Tyr His Ser Gln Lys
        155                 160                 165

ACA GGA AAA TAT TAC GCG ATG GTG ACA GGA AAA GAG GGT GAA TTT GAA      642
Thr Gly Lys Tyr Tyr Ala Met Val Thr Gly Lys Glu Gly Glu Phe Glu
    170                 175                 180

CAA TAC GAA TTA AAG GCG GAC AAA AAT GGA TAC ATA TCC GGC AAA AAG      690
Gln Tyr Glu Leu Lys Ala Asp Lys Asn Gly Tyr Ile Ser Gly Lys Lys
185                 190                 195                 200
```

```
GTA CGG GCG TTT AAA ATG AAT TCC CAG ACG GAA GGG ATG GCA GCA GAC      738
Val Arg Ala Phe Lys Met Asn Ser Gln Thr Glu Gly Met Ala Ala Asp
                205                 210                 215

GAT GAA TAC GGC AGG CTT TAT ATC GCA GAA GAA GAT GAG GCC ATT TGG      786
Asp Glu Tyr Gly Arg Leu Tyr Ile Ala Glu Glu Asp Glu Ala Ile Trp
            220                 225                 230

AAG TTC AGC GCC GAG CCG GAC GGC GGC AGT AAC GGA ACG GTT ATC GAC      834
Lys Phe Ser Ala Glu Pro Asp Gly Gly Ser Asn Gly Thr Val Ile Asp
        235                 240                 245

CGT GCC GAC GGC AGG CAT TTA ACT CGT GAT ATT GAA GGA TTG ACG ATT      882
Arg Ala Asp Gly Arg His Leu Thr Arg Asp Ile Glu Gly Leu Thr Ile
    250                 255                 260

TAC TAC GCT GCT GAC GGG AAA GGC TAT CTG ATG GCA TCA AGC CAG GGA      930
Tyr Tyr Ala Ala Asp Gly Lys Gly Tyr Leu Met Ala Ser Ser Gln Gly
265                 270                 275                 280

AAC AGC AGC TAC GCC ATT TAT GAC AGA CAA GGA AAG AAC AAA TAT GTT      978
Asn Ser Ser Tyr Ala Ile Tyr Asp Arg Gln Gly Lys Asn Lys Tyr Val
                285                 290                 295

GCG GAT TTT CGC ATA ACA GAC GGT CCT GAA ACA GAC GGG ACA AGC GAT     1026
Ala Asp Phe Arg Ile Thr Asp Gly Pro Glu Thr Asp Gly Thr Ser Asp
            300                 305                 310

AC[redacted]A ATT GAC GTT CTG GGT TTC GGA CTG GGG CCT GAA TAT CCG     1074
Th[redacted]y Ile Asp Val Leu Gly Phe Gly Leu Gly Pro Glu Tyr Pro
        315                 320                 325

TTC GGT ATT TTT GTC GCA CAG GAC GGT GAA AAT ATA GAT CAC GGC CAA     1122
Phe Gly Ile Phe Val Ala Gln Asp Gly Glu Asn Ile Asp His Gly Gln
    330                 335                 340
```

```
AAG GCC AAT CAA AAT TTT AAA ATC GTG CCA TGG GAA AGA ATT GCT GAT      1170
Lys Ala Asn Gln Asn Phe Lys Ile Val Pro Trp Glu Arg Ile Ala Asp
345                 350                 355                 360

CAA ATC GGT TTC CGC CCG CTG GCA AAT GAA CAG GTT GAC CCG AGA AAA      1218
Gln Ile Gly Phe Arg Pro Leu Ala Asn Glu Gln Val Asp Pro Arg Lys
                365                 370                 375

CTG ACC GAC AGA AGC GGA AAA TAAACATGCA AAAAGCAGCT TATACAAGCT         1269
Leu Thr Asp Arg Ser Gly Lys
            380

GCTTTTTGCA TGTGAAGAAC G                                              1290
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 383 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Asn His Ser Lys Thr Leu Leu Leu Thr Ala Ala Ala Gly Leu Met
 1               5                  10                  15

Leu Thr Cys Gly Ala Val Ser Ser Gln Ala Lys His Lys Leu Ser Asp
            20                  25                  30

Pro Tyr His Phe Thr Val Asn Ala Ala Ala Glu Thr Glu Pro Val Asp
        35                  40                  45

Thr Ala Gly Asp Ala Ala Asp Asp Pro Ala Ile Trp Leu Asp Pro Lys
    50                  55                  60
```

Thr Pro Gln Asn Ser Lys Leu Ile Thr Thr Asn Lys Lys Ser Gly Leu
65 70 75 80

Val Val Tyr Ser Leu Asp Gly Lys Met Leu His Ser Tyr Asn Thr Gly
85 90 95

Lys Leu Asn Asn Val Asp Ile Arg Tyr Asp Phe Pro Leu Asn Gly Lys
100 105 110

Lys Val Asp Ile Ala Ala Ala Ser Asn Arg Ser Glu Gly Lys Asn Thr
115 120 125

Ile Glu Ile Tyr Ala Ile Asp Gly Lys Asn Gly Thr Leu Gln Ser Met
130 135 140

Thr Asp Pro Asp His Pro Ile Ala Thr Ala Ile Asn Glu Val Tyr Gly
145 150 155 160

Phe Thr Leu Tyr His Ser Gln Lys Thr Gly Lys Tyr Tyr Ala Met Val
165 170 175

Thr Gly Lys Glu Gly Glu Phe Glu Gln Tyr Glu Leu Lys Ala Asp Lys
180 185 190

Asn Gly Tyr Ile Ser Gly Lys Lys Val Arg Ala Phe Lys Met Asn Ser
195 200 205

Gln Thr Glu Gly Met Ala Ala Asp Asp Glu Tyr Gly Arg Leu Tyr Ile
210 215 220

Ala Glu Glu Asp Glu Ala Ile Trp Lys Phe Ser Ala Glu Pro Asp Gly
225 230 235 240

Gly Ser Asn Gly Thr Val Ile Asp Arg Ala Asp Gly Arg His Leu Thr
245 250 255

Arg Asp Ile Glu Gly Leu Thr Ile Tyr Tyr Ala Ala Asp Gly Lys Gly
260 265 270

Tyr Leu Met Ala Ser Ser Gln Gly Asn Ser Ser Tyr Ala Ile Tyr Asp
275 280 285

Arg Gln Gly Lys Asn Lys Tyr Val Ala Asp Phe Arg Ile Thr Asp Gly
290 295 300

Pro Glu Thr Asp Gly Thr Ser Asp Thr Asp Gly Ile Asp Val Leu Gly
305 310 315 320

Phe Gly Leu Gly Pro Glu Tyr Pro Phe Gly Ile Phe Val Ala Gln Asp
325 330 335

Gly Glu Asn Ile Asp His Gly Gln Lys Ala Asn Gln Asn Phe Lys Ile
340 345 350

Val Pro Trp Glu Arg Ile Ala Asp Gln Ile Gly Phe Arg Pro Leu Ala
355 360 365

Asn Glu Gln Val Asp Pro Arg Lys Leu Thr Asp Arg Ser Gly Lys
370 375 380

Claims:

1. Phytase or a functional derivative thereof, characterised in that said phytase has a specific activity of at least 20 U/mg protein, wherein said specific activity is determined by incubating said phytase in a solution containing 100 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, and 1.6 mM sodium phytate at 37°C for 30 minutes.

2. Phytase according to claim 1, characterised in that said phytase has a pH optimum of greater than or equal to pH 6.5, wherein said pH optimum is determined by incubating said phytase in a solution containing 100 mM maleic acid-Tris, 1 mM $CaCl_2$, and 1.6 mM sodium phytate at 37°C for 30 minutes or a pH optimum of greater than or equal to pH 7.0, wherein said pH optimum is determined by incubating said phytase in a solution containing 100 mM Tris-HCl, 1 mM $CaCl_2$, and 1.6 mM sodium phytate at 37°C for 30 minutes or by incubating said phytase in a solution containing wheat bran extract, 1 mM $CaCl_2$, and 1.6 mM sodium phytate at 37°C for 30 minutes.

3. Phytase according to claim 1 or 2, characterised in that said phytase is capable of functioning in the presence of digestive enzymes found in the small intestine of animals.

4. Phytase according to any one of claims 1 to 3, characterised in that the relative activity of said phytase during food or feed processing is greater than or equal to 30% compared to the activity of said phytase in the digestive tract of a farm animal.

5. Phytase according to any one of claims 1 to 4, characterised in that said phytase is obtainable from a microbial source.

6. Phytase according to any one of claims 1 to 5, characterised in that said microbial source is a strain of Bacillus.

7. Phytase according to any one of claims 1 to 6, characterised in that said Bacillus strain is selected from the group comprising Bacillus subtilis and Bacillus amyloliquefaciens.

8. Phytase according to any one of claims 1 to 7, characterised in that said Bacillus strain is Bacillus subtilis strain BS 13 deposited at the National Collections of Industrial and Marine Bacteria, Ltd. (NCIMB) in Scotland under accession number NCIMB-40819.

9. Phytase according to any one of claims 1 to 8, characterised in that said phytase comprises the amino acid sequence according to SEQ ID NO: 1 or a functional derivative thereof.

10. An isolated nucleic acid or a functional derivative thereof which encodes a phytase according to any one of claims 1 to 9.

11. A nucleic acid according to claim 10, characterised in that said nucleic acid comprises a DNA sequence according to SEQ ID NO: 1 or a functional derivative thereof.

12. A nucleic acid according to claim 10, characterised in that said nucleic acid hybridises to a DNA sequence according to SEQ ID NO: 1 or a functional derivative thereof.

13. An isolated nucleic acid which encodes a phytase, characterized in that said nucleic acid hybridises to a DNA according to SEQ ID NO: 1 and encodes a phytase having a pH optimum of greater than or equal to pH 5.0 and a specific activity of at least 10 U/mg protein as determined in a solution containing 100 mM maleic acid-Tris, 1 mM $CaCl_2$, and 1.6 mM sodium phytate at 37°C for 30 minutes.

14. A nucleic acid according to any one of claims 10 to 13, characterised in that said nucleic acid is a DNA molecule.

15. A vector comprising a DNA molecule according to claim 14.

16. A vector according to claim 15, characterised in that said DNA molecule is functionally linked to regulatory sequences capable of expressing a phytase from said DNA sequence.

17. A vector according to claim 16, characterised in that said DNA molecule comprises a leader sequence capable of providing for the secretion of said phytase.

18. A prokaryotic host cell transformed by a nucleic acid or vector according to any one of claims 10 to 17.

19. A prokaryotic host cell according to claim 18, characterised in that said host cell is selected from the group comprising E. coli, Bacillus sp., Lactobacillus sp. and Lactococcus sp.

20. A eukaryotic host cell or organism transformed by a nucleic acid or vector according to any one of claims 10 to 17.

21. A eukaryotic host cell or organism according to claim 20, characterised in that said host cell is selected from the group comprising Aspergillus sp., Humicola sp., Pichia sp., Trichoderma sp. Saccharomyces sp. and plants such as soybean, corn and rapeseed.

22. Method for the recombinant production of phytase, characterised in that a host cell or organism according to any one of claims 18 to 21 is cultured or cultivated under suitable conditions and said phytase is recovered.

23. Use of phytase according to any one of claims 1 to 9 in food or animal feed.

24. Food or animal feed comprising phytase according to any one of claims 1 to 9.

25. Food or animal feed according to claim 24, characterised in that said food or animal feed comprises phytase as an additive which is active in the digestive tract of said animal.

26. Food or animal feed according to claim 24, characterised in that said food or animal feed comprises phytase as an additive which is active in food or feed processing.

27. Method for the production of a food or animal feed according to claim 24 to 26, characterised in that said phytase is sprayed in liquid form onto said food or animal feed.

28. Method for the production of a food or animal feed according to claim 24 to 26, characterised in that said phytase is mixed as a dry product with said food or animal feed.

29. Use of animal feed according to claim 24 to 26 for animals selected from the group comprising avians including poultry, ruminants including bovine and sheep, pig, and aquatic farm animals including fish and shrimp.

30. Use of phytase according to any one of claims 1 to 9 in the production of inositol, inorganic phosphate and phosphorylated intermediates.

31. Method for the reduction of levels of phytate in animal manure, characterised in that an animal is fed an animal feed according to 24 to 26 in an amount effective in converting phytate contained in said animal feed.

32. Method for the production of a nucleic acid which encodes a phytase according to any one of claims 1 to 9, characterised in that a probe comprising a nucleic acid according to any one of claims 10 to 13 is hybridised to a sample suspected of containing said nucleic acid and said nucleic acid is recovered.

33. Use of prokaryotic cells or spores capable of expressing phytase according to any one of claims 1 to 9 as a probiotic or direct fed microbial.

34. Food or animal feed comprising prokaryotic cells or spores capable of expressing phytase according to any one of claims 1 to 9.

35. Method for the production of a food or animal feed, characterised in that prokaryotic cells and/or spores capable of expressing phytase according to any one of claims 1 to 9 are added to said food or animal feed.

The Patent Office

39

Application No: GB 9616957.8 **Examiner:** Colin Sherrington
Claims searched: 1 to 35 **Date of search:** 22 October 1996

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.O): C3H(HC1)

Int Cl (Ed.6): C12N 9/16,

Other: ONLINE: WPI,CLAIMS,DIALOG/BIOTECH,CAS ONLINE,DGENE

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
A	Derwent WPI Abstract Accession No. 94-094823/12 & JP060038745A	1, 2, 7, 10
A	Biosci.Biotech.Biochem. 1992,56(8),1266-1269 -Mikio Shimizu "Purification and Characterization of Phytase from *Bacillus subtilis* (*natto*) N-77"	1, 2, 7, 10
A	J.Bacteriology 1982,151(3),1102-1108 -Vishnu K.Powar *et al.* "Purification and Properties of Phytate-Specific Phosphatase from *Bacillus subtilis*"	1, 2, 7, 10

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

(19) 대한민국특허청(KR)
(12) 공개특허공보(A)

(51) Int. Cl.[6]
C12N 15/52

(11) 공개번호 특1999-0086028
(43) 공개일자 1999년12월15일

(21) 출원번호 10-1998-0018810
(22) 출원일자 1998년05월25일

(71) 출원인 주식회사 우진 강석진
경기도 화성군 양감면 송산리 528-5

(72) 발명자 배희동
경기도 수원시 팔달구 우만동 주공2단지아파트 201동 404호

포스버그 세실 더블유
디파트먼트 오브 마이크로바이올로지 유니버서티 오브 구엘프 구엘프, 온타리오 엔1지 2더블유1,카나다

골로벤 세르게이
디파트먼트 오브 마이크로바이올로지 유니버서티 오브 구엘프 구엘프, 온타리오 엔1지 2더블유1,카나다

청 규오 조안
애그리컬쳐 앤드 애그리-풋 카나다 리서치 스테이션 피.오. 박스 3 메인 리 스브리지, 알베르타 티1제이 4비1,카나다

(74) 대리인 이덕룩

심사청구 : 있음

(54) 신규한 파이타제 유전자, 재조합 파이타제 및 그 용도

요약

본 발명은 고활성의 우수한 파이타제 유전자 PhyE의 염기서열을 결정하고 이유전자로 대장균 BL21(DE3)를 형질전환시킨 후 배양하여 재조합 파이타제를 대량 생산하는 방법을 제공한다.

본 발명의 재조합 파이타제는 넓은 pH 범위에서 안정하고 4 내지 60℃의 온도에서 역가가 우수하며 단백질 분해효소에 대한 저항성이 뛰어나 가축사료 첨가물로 이용할 경우 가축의 성장을 촉진하고 가축 분내의 인의 함량을 감소시켜 환경오염을 감소시키는 뛰어난 효과가 있다.

대표도

도2

명세서

도면의 간단한 설명

도 1은 대장균 ATCC 33965로부터 appA 유전자를 클로닝한 결과를 나타낸다.

도 2는 파이타제 활성이 우수한 PhyE 유전자의 염기서열을 나타낸다.

도 3은 파이타제 활성이 우수한 PhyE 유전자의 아미노산 서열을 나타낸다.

도 4는 파이타제 활성이 있는 대장균 클론을 스크링한 결과를 나타낸다.

도 5는 여러 단계의 정제를 거친 재조합 파이타제의 SDS-PAGE 분석 사진도이다.

도 6은 세포추출물내의 단백질을 pH2.5에서 산침전시켜 정제한 재조합 파이타제를 SDS-PAGE로 분석한

사진도이다.

도 7은 위액과 판크레틱 프로테아제(pancreatic protease)에 대한 파이타제 저항성을 나타낸 그래프이다.

도 8은 대장균 파이타제활성에 미치는 pH의 영향을 나타낸 그래프이다.

도 9는 대장균 파이타제의 최적 온도를 나타낸 그래프이다.

도 10은 재조합 파이타제 활성을 한스-울프 플롯(Hanes-Woolf plot)으로 나타낸 그래프이다.

발명의 상세한 설명

발명의 목적

발명이 속하는 기술 및 그 분야의 종래기술

본 발명은 신규한 파이타제 효소에 관한 것이다. 더욱 상세하게는, 활성이 우수한 파이타제 효소의 유전자를 결정하고 클로닝한 후 숙주세포에 도입하여 활성이 우수한 파이타제 효소를 생산하는 방법에 관한 것이다.

인은 식물에 있어서 필수 광물질로서 가축 사양시 세 번째로 많이 요구되는 성분이다. 일반적으로 두류나 곡류에 함유되어 있고 전체 인 가운데 60 ~ 70%는 피틴산(Phytic acid; inositol hexaphosphate)으로 불리우는 유기물 형태로 존재하며 주로 1가 혹은 2가의 양이온 파이테이트(phytate)염 형태로서 식물내 함량은 0.4 ~ 0.6%로 알려져 있다. 그러나 이러한 파이테이트는 단위가축이 소화흡수하지 못하고 분으로 배설하므로써 인의 환경오염원에 직접적인 영향을 미치는 심각한 물질로 알려져 있다. 또한 성장을 위하여 인을 필요로 하는 단위가축은 파이테이트를 이용하지 못하기 때문에 가축의 생산성 유지 또는 개선을 위하여 무기태인(inorganic phosphate)의 사료내 첨가가 필수불가결 하였다. 이러한 무기태인의 사료내 첨가는 가금류나 돼지의 생산성을 향상시키지만 경제적으로 가축사육 농민들에게는 상당한 경제적 부담을 주게됨은 물론 분으로 배설되는 파이테이트는 환경오염 물질로 배출이 규제되고있다.

따라서 이러한 문제를 해결하기 위하여 네덜란드, 독일, 한국 및 대만정부는 돼지나 가금류의 분에 포함된 인으로 인해 발생하는 환경오염을 입법으로 막고 있거나 서두르고 있다. 축산업계에서는 인의 공급을 감소시키고 오염을 줄이기 위한 방법을 다각도로 모색하고 있으며 그중 한 방법으로서 사료에 함유되어 있는 파이테이트내 인의 이용율을 높히므로써 인의 첨가량을 감소시킬 수 있는 파이타제(Phytase)의 첨가 방법을 가장 바람직한 것으로 검토하고 있고 이를 입법화하려는 경향이 있다.

최근 양계 및 양돈 사료에 파이타제를 첨가하여 파이테이트 이용율을 높히고 인으로 인한 오염을 예방하려는 연구가 진행되고 있다. 파이타제는 파이테이트를 무기태인과 인노시톨(inositol)로 가수분해하므로써 돼지에서 인 이용율을 24%까지 증가시키고 그만큼 사료내의 인 첨가량을 감소시킨다고 알려져 있다. 즉, 인의 이용성을 증가시키므로써 분내 35%의 인 배설량을 감소시키고 분내에 함유된 인으로 인한 환경오염을 그만큼 감소시킬 수 있다. 또한 사료내 파이타제를 첨가하면 무기태인의 첨가량을 감소시킬 수 있으며 파이타제는 칼슘이나 아연과 같은 광물질의 생물학적 이용율을 증가시키고 파이테이트와 결합되어 있거나 킬레이트된 아미노산과 같은 영양소의 이용율을 증가시켜 이용성을 현저히 개선시키는 효과가 있어 결국 사료의 이용성을 현저히 개선시키는 것으로 알려져 있다. 최근에는 아스퍼지러스 나이거(Aspergillus niger) 또는 아스퍼질러스 피쿠엄(A. ficuum)등의 곰팡이로부터 사료용 파이타제가 생산되어 이용되고 있다. 아스퍼질러스 나이거의 파이타제는 최적 pH가 2.5 ~ 5.5에 이르며 돼지나 가금류의 위나 사낭에서 효소역가를 나타내지만 pH가 6이상인 소장내에서는 어려운 것으로 보고되고 있어 이를 보완할 방법이 요구되어 왔다. 그리고 아스퍼질러스 나이거 유래의 파이타제 비활성(Specific activity)은 약 100unit 정도인데 이러한 정도의 역가는 그렇게 높은 것이 아니기 때문에 비용절감을 위하여 이보다 훨씬 높은 파이타제 개발이 요구되고 있다.

파이타제는 옥수수, 캐놀라 등 광범위한 식물이나 프로토조아(protozoa), 마이커라이저(mycorrhizae), 곰팡이, 이스트 및 박테리아 등에서 생산된다. 특히 보고된 모든 파이타제는 대개 8.7unit(B. substillis)에서 63unit(K.aerogens)에 이르기까지 그렇게 높은 활성을 나타내지 않으나 대장균 스트레인 K12(E.coli strain K12)가 8000unit에 이르는 높은 활성을 나타내는 것으로 보고되어 있으며, Km과 k_{cat} 는 0.13mM과 6209s^{-1}로 알려져 있다. 대장균의 경우 인산화되어 있는 물질을 분해하는 능력을 지닌 애시드 포스파타제(acid phosphatase)도 약 750(U/mg protein) 정도이고 Km과 k_{cat}는 7.8mM과 490s^{-1}로 알려져 있다. 또한 대장균 파이타제의 분자량은 다른 미생물로부터 생산되는 파이타제보다 현저히 낮은 약 42,000이고 모노머로서 기능을 가지고 있으며 애시드 포스파타제는 최적 pH가 2.5였던 반면에 파이타제는 다른 미생물이 지니고 있는 바와 같이 최적 pH는 4.5이고 최하로 pH 2.0까지 활성을 나타내며 4℃에서 pH 1.0에 24시간 노출했을 경우에도 약 30%의 활성을 나타냈고 효소의 최적 온도는 55℃이고 60℃에서 급격히 감소하는 특징을 지닌 것으로 알려져 있다. 또한 대장균 파이타제는 50℃에서 1시간동안 안정적이고 60℃에서 약 24%만 유지되고 70℃에

서는 거의 활성을 나타내지 않는 것으로 보고되었다. 그러나, 대장균의 이러한 파이타제 유전자는 지금까지 클로닝된 바 없으며, 이를 연구한 Greiner 등(1993)은 Dassa 등(1982)에 의하여 파이타제는 최적 pH가 2.5인 애시드 포스파타제와 유사한 것이라고 결론지었다.

본 발명자는 대장균 계통(E.coli) K10의 appA 유전자에 의하여 암호화된 애시드 포스파타제가 매우 높은 파이타제 활성을 나타내므로 향후 사료뿐만 아니라 다른 산업에서도 활용할 가능성이 매우 높은 것에 주목하여 본 발명을 완성하기에 이르렀다.

따라서, 본 발명의 목적은 고활성의 파이타제 효소를 대장균에서 발현되는 포스파타제로부터 분리정제함에 있다. 본 발명의 다른 목적은 고활성의 파이타제를 암호화하는 유전자를 시퀀싱한 다음 이 유전자로 숙주세포를 형질전환시킨 후 배양하여 고활성의 파이타제를 대량 생산함에 있다.

발명이 이루고자하는 기술적 과제

본 발명의 상기 목적은 대장균(E.coli)이 가지고 있으며 appA유전자에 의해 파이타제활성을 나타내는 효소 중 가장 우수한 파이타제활성을 나타내는 효소를 스크링하고 이 효소의 유전자 염기서열을 결정한 후 PhyE 유전자를 PCR로 증폭하고 pET 21a(+) 벡터에 삽입하여 숙주세포를 형질전환시켜 배양하므로서 재조합된 파이타제를 발현시키고 발현된 재조합 파이타제를 농축하고 정제한 후 SDS-PAGE 분석하고 단백질을 정량한 다음 파이타제와 애시드 포스파타제 활성을 측정한 후 이어서 재조합 파아타제 분획을 단백질 분해효소가 함유된 단백질 혼합액에 첨가하고 효소활성을 측정하여 단백질 분해효소에 대한 저항성, 최적 온도, 최적 pH, 반응속도 등 효소특성을 조사하므로써 달성하였다.

발명의 구성 및 작용

본 발명은 대장균이 가지는 최소한 6가지의 페리플라스믹 포스파타제(periplasmic phosphatases; Wanner, 1996)를 제한효소로 처리하고 플라스미드에 삽입한 후 이 플라스미드로 대장균 DH5α를 형질전환시키고 배양하는 단계; 배양된 콜로니의 파이타제 활성 및 애시드 포스파타제 활성을 측정하여 파이타제 활성이 가장 우수한 효소를 얻은 후 이 효소의 유전자 염기서열을 결정하고 PhyE로 명명하는 단계; PhyE 유전자를 벡터에 삽입하고 이 벡터로 대장균 BL21(DE3)을 형질전환시킨 후 배양하여 PhyE 유전자에 의해 재조합된 파이타제를 발현시키는 단계; 발현된 재조합 파이타제를 추출하고 농축한 후 DEAE-세파로스 CL 6B 및 세파덱스 G-75 칼럼으로 크로마토그래피하여 정제하는 단계; 정제된 재조합 파이타제를 SDS-PAGE로 분석한 후 단백질을 정량하는 단계; 재조합 파이타제의 파이타제활성 및 애시드 포스파타제활성을 측정하는 단계; 단백질과 단백질 분해효소가 함유된 혼합액에 정제된 재조합 파이타제 효소를 첨가하고 효소활성을 측정하여 단백질분해효소에 대한 저항성을 측정하는 단계; 온도와 pH를 달리하면서 재조합 파이타제 효소의 활성 변화를 측정하여 최적 온도 및 최적 pH를 조사하는 단계 및; 효소의 반응속도를 조사하는 단계로 구성된다.

본 발명의 실험에 사용된 박테리아 균주는 대장균(E.coli)계통으로 DH5α([supE44$^{\Delta}$ *lacU*169(Φ80 *lacZ* $^{\Delta}$M15_ *hsd*R17 *rec*A1 *end*A1 *gry* A96thi -*rel*A1])(Hananhan 1983), CU1867(appA:Kan^{R})(Ostanin 등., 1992)와 BL21(DE3)(Novagen, Madison, WI) 및 BL21(DE3)(F' *ompT* *hsd*S_B(r_B'm_B') *dcm gal*(DE3)(Novagen, Madison, WI) 등이다. 또 모든 분자생물학적 방법은 샘블럭(Sambrooke;1989)의 방법에 의하여 수행하였다. DNA는 CTBA 처리없이 수행하는 것만 제외하고 머레이(Mrray)와 톰슨(Thomson)(1980)의 방법에 의하여 수행하고 DNA의 PCR 증폭은 사이키(Saiki) 등(1988)의 방법에 의하여 수행하였다.

이하, 본 발명의 구체적인 구성 및 작용을 실시예를 들어 상세히 설명하지만 본 발명의 권리범위는 이들 실시예에만 한정하는 것은 아니다.

<u>실시예 1: 파이타제활성 효소 스크링 및 유전자 염기서열 결정</u>

실험예 1: 대장균 ATCC 33965로부터 파이타제 활성효소 스크링

대장균은 여러종류의 포스파타제를 가지고 있으며 최소한 6가지의 페리플라스믹 포스파타제(Wanner, 1996)가 존재하고 있는 것으로 알려져 있다. 본 발명자는 돼지와 같은 단위동물의 위내 산성조건내에서 활성이 있는 파이테이트-분해 효소(phytate-degrading enzyme)를 대장균 ATCC 33965 로부터 스크링하였다. 이때 사이토플라스믹 효소(Cytoplasmic enzyme)보다는 페리플라스믹 효소(periplasmic enzyme)가 안정적이므로 페리플라스믹 효소를 사용하였다.

대장균 ATCC 33965로부터 채취한 게놈 DNA는 제한효소 BamHI과 Bg1II 엔도뉴클레이제(endonucleases)로 절단하여 4-6kb의 분획을 아가로스 겔(agarose gel)로 추출한 후 constitute 프로모터인 tet gene를 사용하여(Boguet 등, 1987) 모든 유전자가 발현되는 pBR 322 플라스미드의 BamHI 부위에 라이게이션시킨 후, 이 벡터로 대장균 DH5α스트레인을 형질전환시키고 엠피실린이 함유된 LB 배지에서 배양하였

다.

실험예 2: 파이타제 및 애시드포스파타제 활성측정

appA유전자에 의해 애시드 포스파타제활성을 나타낼 경우 도 1에서 나타내는 바와 같이 검은 침전을 형성하는 염색약(0.1M 소듐 아세테이트 pH 5, 0.1% α-나프틸 포스페이트, 0.2% fast Garnet GBC 염, 37℃)을 상기 실험예 1에서 배양한 콜로니에 처리하여 애시드 포스파타제를 스크링하여 선별한 후 높은 애시드 포스파타제 역가를 나타내는 콜로니를 다시 플레이트에서 배양하였다. 배양된 상기 콜로니를 파이테이트가 함유된 아가(1% 아가, 10mM 소듐 아세테이트 pH 4.5, 20mM 소듐 파이테이트, 50mM $CaCl_2$) 배치 상층에 오버레이(overlay)하고 6시간 배양한 후 파이타제 활성을 측정하여 파이타제 활성이 우수한 콜로니를 선별하였다. 실험결과, Greiner 등(1993)이 추출한 효소(애시드 포스파타제 활성: 790unit/mg, 파이타제 활성: 8000 unit/mg)보다도 낮은 애시드 포스파타제 활성(1200 unit/mg)을 나타냈다. 브래드포드 방법으로 측정된 단백질 함량을 기준으로 계산하더라도 greiner 등(1993)의 결과와는 차이가 있었다(애시드 포스파타제 활성: 12,883 unit/mg, 파이타제 활성: 38,649 unit/mg). 또한 파이타제/애시드 포스파타제 비율 차이도 각각 독립적인 방법을 취하였을 때 Greiner 등이 10배였던 것에 비해서 3배의 차이를 나타냈다.

실험예 3: 파이타제 활성효소 유전자 염기서열 결정

상기 실험예 2의 결과 선발한 우수한 파이타제 활성 콜로니를 제한맵핑(restriction mapping)에 따라 3개의 유전자 그룹으로 분류했다. 실험결과, 가장 파이타제활성이 우수한 효소 유전자인 PhyE와 상기 PhyE 유전자와는 다른 특성을 지닌 효소 유전자 Agp 및 기능이 밝혀지지 않은 효소 유전자 PhyC 유전자로 분류하였다. PhyE 유전자의 염기서열은 도 2에 나타냈고 아미노산 서열은 도 3에 나타냈다.

실시예 2: PhyE 유전자의 PCR 및 형질전환

상기 실시예 1의 실험예 3에서 얻은 PhyE 유전자는 하기와 같은 올리고뉴클레오타이드 프라이머 쌍을 사용하여 PCR로 증폭하였다.

APPA-ATG 5'GCATATGAAAGCGATCTTAACCCAT 3'(NdeI site)

APPA-TAA 5'GGGAATTCATTACAAACTGCACGCCG 3'(EcoR1 site)

PCR 산물을 적절한 엔도뉴클레이즈(endonuclease)로 절단한 후, Nde1과 EcoR1 엔도글루카네이즈(endoglucanase)로 처리한 pET21a(+) 발현벡터(Novagen, Madision, WI)에 라이게이션시켰다. 이 발현벡터로 대장균 BL21(DE3)를 형질전환시키고 엠피실린이 함유된 LB 배지에서 배양하였다. 37℃에서 12시간 배양한 플레이트는 파이테이트가 함유된 배지에 오버레이(overlay)시켰다. 도 4와 같이 할로스(Halos) 생성 콜로니는 분석용으로 보존하였다.

본 실시예에서 형질전환된 대장균 BL21(DE3)은 한국종균협회에 1998년 5월 13일자로 기탁번호 KFCC 11033로 기탁하였다.

실시예 3: 재조합 파이타제 효소의 정제

실험예 1: 표준방법에 의한 파이타제 정제

재조합 파이타제는 Ostanin 등(1992)의 방법을 수정하여 추출, 정제하였다. 실시예 2에서 12시간 배양한 대장균 BL21(DE3)을 엠피실린(100μg/mL)을 첨가한 4리터 LB 배지에 접종하여 37℃에서 24시간 셰이킹(Shaking) 배양하였다. 세포를 수거한 후 차가운 사린(saline)(0.82%) 용액으로 세척하고 1mM EDTA 및 라이소자임(lysozyme)(1㎎㎖)이 함유된 200mL 차가운 20%(W/V)슈크로스 용액[33mM Tris/HCl(pH 8.0)에 다시 재현탁시키고 20분간 방치한 후 15분간 8000rpm에서 원심분리하여 상등액을 채취하였으며 pM30 막(Amicon)을 통하여 한외여과(Utrafiltration)시켜 농축하였다. 상기 모든 과정은 4℃에서 수행되었으며 농축시킨 상등액은 50mM Tris/HCl(pH 7.8) 완충용액으로 미리 균형을 맞춘 DEAE-세파로스 CL-6B 칼럼(2.6 x 30cm)에 옮겨 분리하고 800mL 완충용액으로 세척한 후 0 ~ 0.2 M 소듐 크로라이드(linear gradient)를 사용하여 약 30mL/h 속도로 용출시켰다. 이때 효소는 0.09 ~ 0.1 M NaCl 조건에서 용출시켰다. 가장 높은 활성을 나타낸 분획과 단백질 함량이 가장 낮은 분획은 다시 혼합한 후 PM30 막(Amicon)에 다시 통과시켜 농축하였다. 농축된 용액은 50mM Tris/HCl(pH 7.8) 완충용액으로 미리 균형을 맞춘 세파덱스 G-75 칼럼(2.6 x 90cm)에 로딩하였으며 0.9% NaCl과 동일한 완충용액을 사용하여 약 10mL/h 속도로 용출시켰다. 분획은 다시 채취하였으며 재조합 파이타제를 분석하였고 10% SDS-PAGE 겔로 분석하였다. 가장 높은 활성을 나타내고 단백질이 오염되지 않은 분획은 농축하고 PM30 막(Amicon)을 통하여 무석하였다. 정제한 재조합 파이타제는 도 5에 나타낸 바와 같이 10% SDS-PAGE로 분석하였다.

실험예 2: 산 침전에 의한 파이타제 정제

실시예 2에서 4리터 LB 배지에서 배양된 세포를 수거한 후 차가운 사린(saline)(0.82%) 용액으로 세척하고 1mM EDTA 및 라이소자임(lysozyme)(1mg/mL)이 함유된 200mL 차가운 20%(W/V)

슈크로스용액(30mM Tris/HCl)(pH 8.0)에 다시 재현탁시킨 후 세포는 약 20분간 얼음에 방치하였으며 0.1M 글리신/HCl buffer(pH 2.5) 100mL를 첨가하고 얼음에서 약 1시간 가량 방치하였다. 추출된 단백질과 핵산은 약 10,000rpm에서 10분간 원심분리한 후 제거하였으며 채취한 효소 용액은 투석하고 농축시킨 후 DEAE-셀파로스 CL 6B와 세파덱스 G-75에 칼럼 크로마토그래피하여 정제하였다. 정제후 SDS-PAGE로 분석한 결과를 도 6에 나타냈다.

실시예 4: 재조합 파이타제 효소의 단백질 정량

상기 실시예 3에서 정제한 재조합 파이타제의 단백질 함량을 측정하였다. 단백질 함량은 브래드포드 방법(Bradford, 1976), 비시콜니닉 애시드(bicichoninic acid;BCA)를 사용하는 방법(Smith 등, 1985), UV 흡광도를 측정하는 방법(Ostanin 등, 1992) 및 동결건조 후 건물중량을 측정하는 방법으로 측정하였다. 실험결과, 브래드포드 단백질 측정 방법으로 파이타제의 특정 활성이 지나치게 높아 단백질 함량은 소량으로 평가되었다. 단백질 함량 평가방법을 상호비교했을 때, 브래드포드 분석법이 동결건조 방법으로 측정한 수치보다는 낮은 수치를 나타냈으며 OD_{280}nm 측정법이나 비시콜니닉 애시드 방법은 동결건조 방법으로 측정한 수치와 유사한 것으로 나타났다. 이러한 결과를 표1에 정리하였다.

[표 1]

정제된 파이타제의 농도측정

단백질 측정방법	단백질 함량 (mg/mL)	파이타제의 특정활성 (U/mg)	파이타제의 특정활성 (%)
브래드포드(Bio-Red)	0.77	38649	2312
BCA	24.8	1200	72
OD_{280}	14.7	2024	121
건조물량	17.8	1672	100

실시예 5: 재조합 파이타제 효소활성 및 애시드 포스파타제 활성 측정

파이타제 및 애시드 포스파타제활성 측정은 약 37℃에서 측정하였다. 애시드 포스파타제 분석은 p-니트로페닐 포스페이트(25mM)를 기질로 사용하였으며 0.25M 글리신/HCl buffer(pH 2.5) 200㎕에서 10분간 반응시킨 후 1.0M 소듐 하이드로사이드 1mL 첨가하여 반응을 종료시켰으며 유리된 p-니트로페닐 포스페이트 함량은 410nm에서 흡광도를 측정하여 계산하였다. 애시드 포스파타제 역가는 분당 유리된 p-니트로페닐 1μmole을 1unit로 하였다. 파이타제 분석은 소듐 파이테이트(4mM)를 기질로 하여 0.1M 아세테이트(acetate)/아세틱 애시드 버퍼(acetic acid buffer)(pH 4.5) 200㎕에서 10분간 반응시킨 후 133㎕의 암모니움 몰리브데이트(ammonium molybdate):암모니움 반나데이트(ammonium vanadate):니트릭 애시드 (nitric acid) 혼합물을 첨가하여 반응을 정지시켰으며 유리된 무기 포스페이트(inoganic phosphate) 함량은 410nm에서 흡광도를 측정하여 계산하였다. 파이타제 활성은 분당 유리된 무기 포스페이트 1μmole을 1unit로 하였으며 모든 분석은 3번 반복하였다. 실험결과, 파이타제와 애시드 포스파타제의 활성은 단백질 mg당 각각 1,800 unit와 479 unit였으며 파이타제와 애시드 포스파타제활성 비율은 3.8이였다. 이는 실시예 1의 실험예 2에서 ATCC 33965로부터 얻은 결과와 유사하였다. 그러나 대장균 ATCC 33965로 얻은 PhyE 유전자의 염기서열은 appA 유전자 염기서열과 다른 QEPELKLESVV로 확인되었다.

실시예 6: 단백질 분해효소에 대한 저항성 측정

1㎎/mL의 펩신(20mM 글리신/HCl, pH 2.5)이나 각각 1㎎/mL 키모트립신(chymotrypsin), 트립신(trypsin), 엘라스타제(elastase) 및 판크레틴(pancreatin)(20mM MOPS, pH7.0)이 혼합되어 있는 판크레틱 프로테아제(pancretic proteases)의 혼합액에 정제된 재조합 파이타제를 첨가하고 37℃에서 2시간 동안 배양한 후 활성을 측정하여 평가하였다. 또 쥐의 장내 소화액과 위액에 대하여도 같은 방법으로 실험을 실시하였다. 실험결과, pH 2.5인 펩신은 80%의 활성을 나타내 단백질 분해효소에 대해 안정성이 높았다. pH 7인 판크레틱에서는 38%의 활성을 나타내 비교적 좋은 안정성을 나타냈으며(도 7) 쥐의 위액에서는 100% 활성을 나타냈고 장내 소화액에서는 37℃에서 40% 의 역가를 유지하므로써 우수한 안정성이 있음을 확인하였다.

실시예 7: 최적 온도 및 최적 pH 조사

재조합 파이타제의 최적온도를 측정하기 위하여 30℃에서부터 90℃에 걸쳐 표준 파이타제 분석방법을 실시하였다. 최적 pH는 글리신/HCl(pH 1~3.5), 소듐 아세테이트, 아세트산(pH 3.5~6), 트리스/아세트산(pH 6~7), 트리스/HCl(pH 7~9), 글리신/NaOH(pH 9~10)을 사용하여 측정하였으며 모든 완충용액의 농도는 0.1M로

하였다. 실험결과 대장균 파이타제는 0.1M 소듐 아세테이트/아세트산 버퍼 pH 4.5에서 한 개의 적정(Optium) pH를 나타냈다. 또한 pH 7 ~ 7.5 조건에서는 활성이 급격히 감소하는 경향을 나타냈고 pH 7.0에서는 거의 75%의 활성을 나타냈으며 pH 2에서는 40%의 활성을 유지하는 것으로 나타났다(도 8).

파이타제의 최적온도는 메조필릭 유기물(mesophilic organism)이 나타내는 일반적인 현상과는 달리 60℃에서 높은 안정성을 나타냈다(도 9). 또한 일반적인 파이타제 분석법을 사용하였을 때 60℃에서의 특정 활성은 37℃에서의 4.2배 높았으며 37℃에서의 파이타제 활성은 75℃에서의 활성과 같은 것으로 나타났다.

실시예 8: 효소반응속도 측정

효소반응 속도에 대한 기질의 농도 영향은 고정된 효소량을 기준으로 하고 표준 파이타제 분석방법을 사용하여 측정(37℃, 0.1M 소듐 아세테이트/아세트산 버퍼 pH 4.5)하였다. 기질의 농도는 0.2mM에서 10mM로하여 단계별로 측정하였다. 결과는 Sigma Plot 2.01 컴퓨터 프로그램을 사용하여 통계적으로 가장 최적의 직선식(Kuchel과 Ralston, 1988)을 나타내는 한스-울프 플롯(Hanse-Woolf plot) 방법으로 파이타제의 Km과 Vmax를 산출하였다. 실험결과, 재조합 파이타제는 Greiner 등(1993)의 파이타제가 1.5mM의 파이테이트 농도에서 역가가 억제되었다는 보고와는 달리 4mM 이상의 기질농도에서 파이타제 활성이 억제된 것으로 나타났다. 재조합 파이타제의 Kinetic 상수도 Greiner 등(1993)과 달리 나타났는데, Km은 0.63mmol/L, Vmax는 2326μmol/min/mg이었다(도 10).

발명의 효과

이상 실시예와 실험예를 통하여 설명한 바와 같이 본 발명은 파이타제활성이 우수한 PhyE 유전자로 대장균 BL21(DE3)를 형질전환시킨 후 배양하여 재조합 파이타제를 얻는 효과가 있으며 이 재조합 파이타제는 우수한 활성을 나타내고 넓은 pH 범위에서 안정하여 가축사료 첨가제로 사용할 경우 가축성장 촉진 및 가축분내 인 함량의 저감효과가 있으므로 축산업 및 환경친화산업상 매우 유용한 발명인 것이다.

(57) 청구의 범위

청구항 1

대장균 ATCC 33965로부터 분리된 하기 파이타제 유전자(PhyE)의 염기서열.

atgaaagcgatcttaatcccatttttatctcttctgattccgttaacccgcaatctgcattcgctcagaggtgagccggagctgaagctggaaa
gtgtggtgattgtcagtcgtcatggtgtgcgtgctccaaccaaggccacgcaactgatgcaggatgtcaccccagacgcatggccaacctg
gccggtaaaactgggttggctgacaccgcgcggtggtgagctaatcgcctatctcggacattaccaacgccagcgtctggtagccgacgg
attgctggcgaaaaagggctgcccgcagtctggtcaggtcgcgattattgctgatgtcgacgagcgtacccgtaaaacaggccgaagcctt
cgccgccgggctggcacctgactgtgcaataaccgtacataacccaggcagatacgtccagtcccgatccgtattttaatcctctaaaaactg
gcgttgccaactggataacgcgaacgtgactgacgcgatcctcagcagggcaggagggtcaattgctgacttttaccgggcatcggcaaa
cggcgttcgcgaactggaacgggtgcttaattttccgcaatcaaacttgtgcctttaaacgtgaggaacaggacgaaagctgttcattaacg
caggcattaccatcggaactcaaggtgagcgccgacaatgtctcattaacccggtgcggtaagcctcgcatcaatgctgacggagatatttct
cctgcaacaagcacagggaatgccggagccgggtgggaaggatcaccgattcacaccagtggaacacctgctaagtttgcataacg
cgcaatttttattgctacaacgcacgccaggagttgccgcagccgcgccaccccgttattagatttgatcaagacagcgttgacgccccat
ccaccgcaaaaacaggcgtatggtgtgacattaccccactcagtgctgtttatcgccggacacgatactaatctggcaaatctcggcggcgc
actggagctcaactggacgcttcccggtcagccggataacacgccgccaggtggtgaactggtgttgaacgctggcgtcggctaagcg
ataacagccagtggattcaggtttccgctggtcttccagacttacagcagatgcgtgataaaacgccgctgtcattaaatacgccgcccgga
gaggtgaaactgacccggcaggatgtgaagagcgaaatgcgcagggcatgttcgttggcaggtttacgcaaatcgtgaatgaagca
cgcatcccggcgtgcagtttgtaa

청구항 2

PhyE 유전자로 형질전환된 대장균 BL21(DE3)(기탁번호 KFCC 11033).

청구항 3

PhyE 유전자로 형질전환된 대장균 BL21(DE3)를 배양하여 발현된 재조합 파이타제 효소.

청구항 4

제3항에 있어서, 상기 재조합 파이타제 효소의 활성이 pH 약 2.5 내지 7.5, 온도 4 내지 60℃에서 안정하며 pH는 4.5에서는 최적 파이타제 역가를 나타내고 pH 2.5에서는 최적의 애시드 포스파타제를 나타냄을 특징으로 하는 재조합 파아타제 효소.

청구항 5

대장균 ATCC 33965로부터 PhyE 유전자를 분리하고

APPA-ATG 5'GCATATGAAAGCGATCTTAACCCAT 3'(NdeI site)

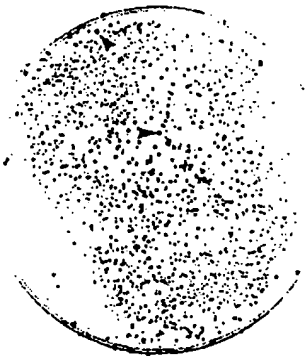
APPA-TAA 5'GGGAATTCATTACAAACTGCACGCCG 3'(EcoR1 site)와 함께 PCR로 증폭한 후 엔도뉴클레이즈로 절단하고 Nde1과 EcoR1으로 처리한 pET21a(+) 발현벡터에 라이케이션시키고 대장균 BL21(DE3)에 도입하여 형질전환시킨 후 배양하여 재조합 파이타제 효소를 생산하는 방법.

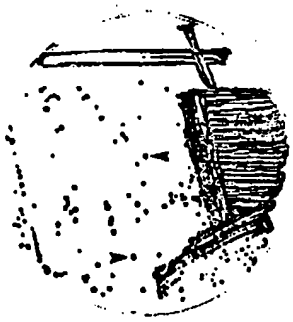
청구항 6

재조합 파이타제 효소를 음수용 물, 미네랄 블록(mineral block), 알약형, 펠렛형 또는 미생물 형태로 가축용 사료에 공급함을 특징으로 하는 가축사료용 첨가제.

도면

도면1





도면2

Information for Seq ID NO:1
Sequence characteristics:
a) Length: 1299 base pairs
b) Type: nucleic acid
c) Strandedness: single
d) Topology: linear

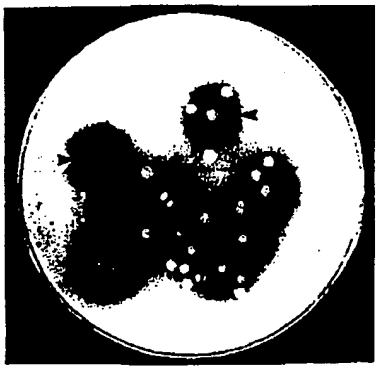
atgaaagcgatcttaatcccattttatctcttctgattccgttaaccccgcaatctgcattcgctcagagtgagccggagctgaagctggaaa
gtgtggtgattgtcagtcgtcatggtgtgcgtgctccaaccaaggccacgcaactgatgcaggatgtcaccccagacgcatggccaacctg
gccggtaaaactgggttggctgacaccgcgcggtggtgagctaatcgcctatctcggacattaccaacgccagcgtctggtagccgacgg
attgctggcgaaaaagggctgcccgcagtctggtcaggtcgcgattattgctgatgtcgacgagcgtacccgtaaaacaggcgaagcctt
cgccgccgggctggcacctgactgtgcaataaccgtacatacccaggcagatacgtccagtcccgatccgttatttaatcctctaaaaactg
gcgtttgccaactggataacgcgaacgtgactgacgcgatcctcagcagggcaggagggtcaattgctgactttaccgggcatcggcaaa
cggcgtttcgcgaactggaacgggtgcttaattttccgcaatcaaacttgtgccttaaacgtgagaaacaggacgaaagctgttcattaacg
caggcattaccatcggaactcaaggtgagcgccgacaatgtctcattaaccggtgcggtaagcctcgcatcaatgctgacggagatatttct
cctgcaacaagcacagggaatgccggagccgggggtggggaaggatcaccgattcacaccagtggaacaccttgctaagtttgcataacg
cgcaatttttatttgctacaacgcacgccagaggttgcccgcagccgcgccacccccgttattagatttgatcangacagcgtgacgccccat
ccaccgcaaaaacaggcgtatggtgtgacattacccactcagtgctgtttatcgccggacacgatactaatctggcaaatctcggcggcgc
actggagctcaactggacgcttcccggtcagccggataacacgccgccaggtggtgaactggtgtttgaacgctggcgtcggctaagcg
ataacagccagtggatcaggtttcgctggtcttccagacttacagcagatgcgtgataaaacgccgctgtcattaaatacgccgcccgga
gaggtgaaactgacccctggcaggatgtgaagagcgaaatgcgcagggcatgtgttcgttggcaggttttacgcaaatcgtgaatgaagca
cgcataccggcgtgcagtttgtaa

도면3

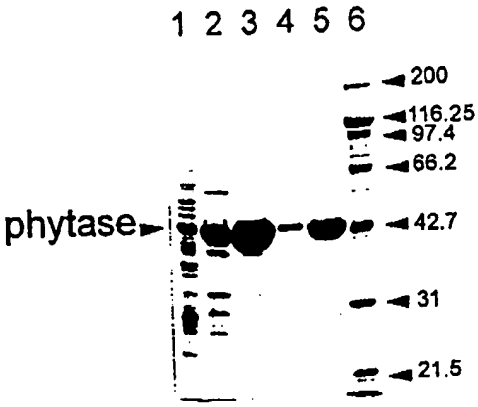
Information for Seq ID NO:2
Sequence characteristics:
a) Length: 432 amino acids
b) Type: protein
c) Topology: linear

MKAILIPFLSLLIPLTPQSAFAQSEPELKLESVVIVSRHGVRAPTKATQLMQ
DVTPDAWPTWPVKLGWLTPRGGELIAYLGHYQRQRLVADGLLAKKGCPQ
SGQVAIIADVDERTRKTGEAFAAGLAPDCAITVHTQADTSSPDPLFNPLKTG
VCQLDNANVTDAILSRAGGSIADFTGHRQTAFRELERVLNFPQSNLCLKREK
QDESCSLTQALPSELKVSADNVSLTGAVSLASMLTEIFLLQQAQGMPEPGW
GRITDSHQWNTLLSLHNAQFYLLQRTPEVARSRATPLLDLIKTALTPHPPQK
QAYGVTLPTSVLFIAGHDTNLANLGGALELNWTLPGQPDNTPPGGELVFER
WRRLSDNSQWIQVSLVFQTLQQMRDKTPLSLNTPPGEVKLTLAGCEERNAQ
GMCSLAGFTQIVNEARIPACSL

도면4

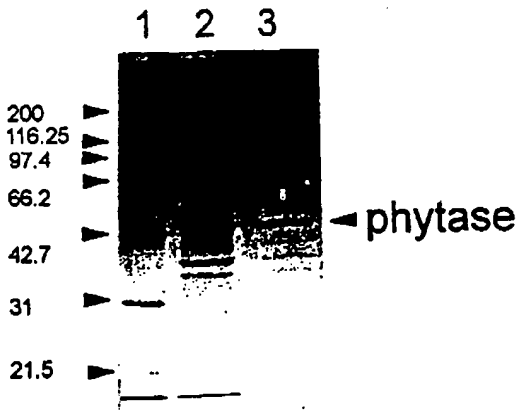


도면5



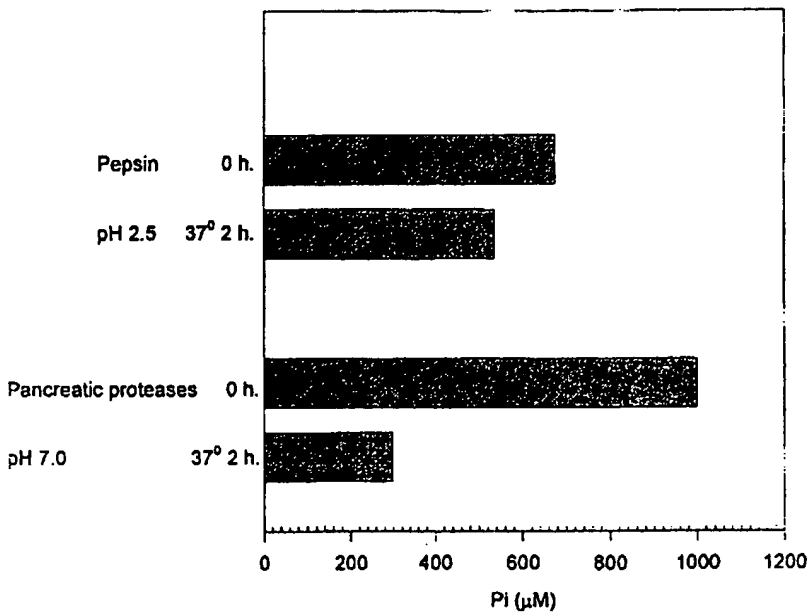
Lane 1. Total cell extract (cells were disrupted with French press).
Lane 2. Periplasmic extract (cells were treated with lysozyme at 1 mg/ml).
Lane 3. After DEAE-Sepharose CL 6B column.
Lane 4-5. After Sephadex G-75 column.
Lane 6. Broad range molecular weight standards (Bio-Rad).

도면6

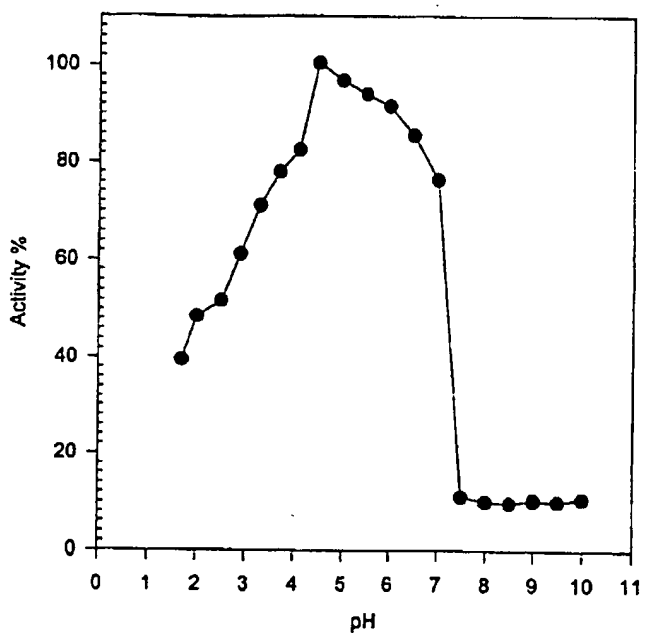


Lane 1. Broad range molecular weight standards (Bio-Rad).
Lane 2. Total cell extract (cells were disrupted with French press).
Lane 3. Acid precipitation of total cell extract.

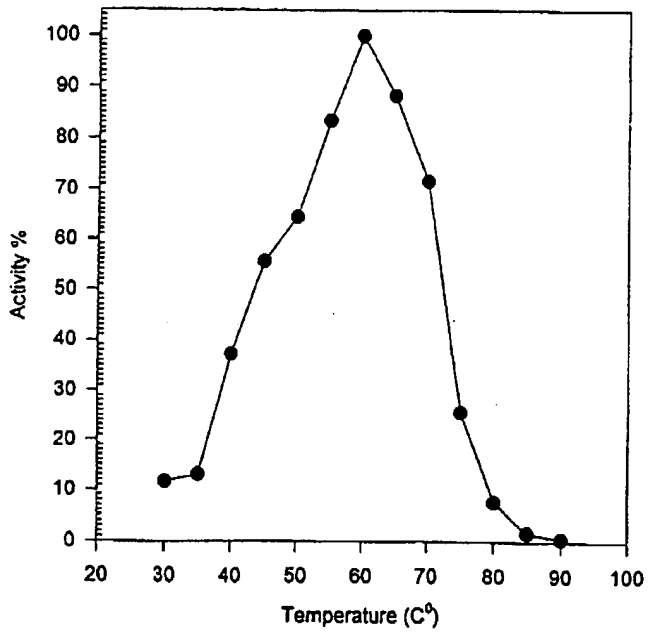
도면7



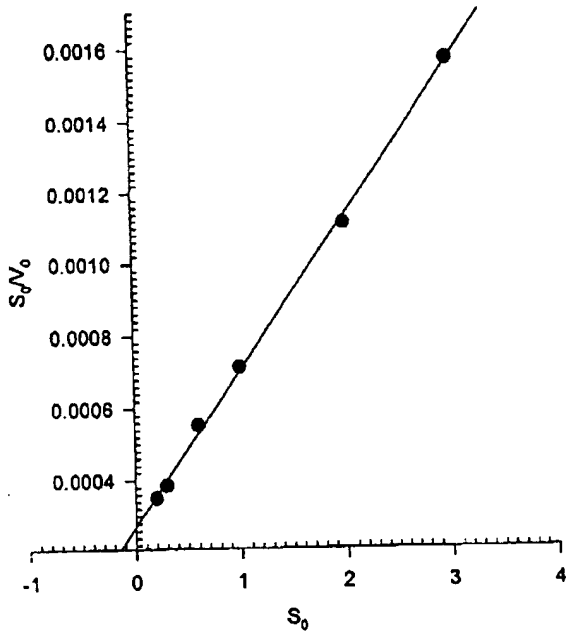
도면8



도면9


Activity %
100
90
80
70
60
50
40
30
20
10
0
20
30
40
50
60
70
80
90
100
Temperature (C°)

도면10



b[0] 2.729 e-4
b[1] 4.299 e-4
r^2 0.99
Km = 0.63 mM,
Vmax = 2326 μmoles/min/mg